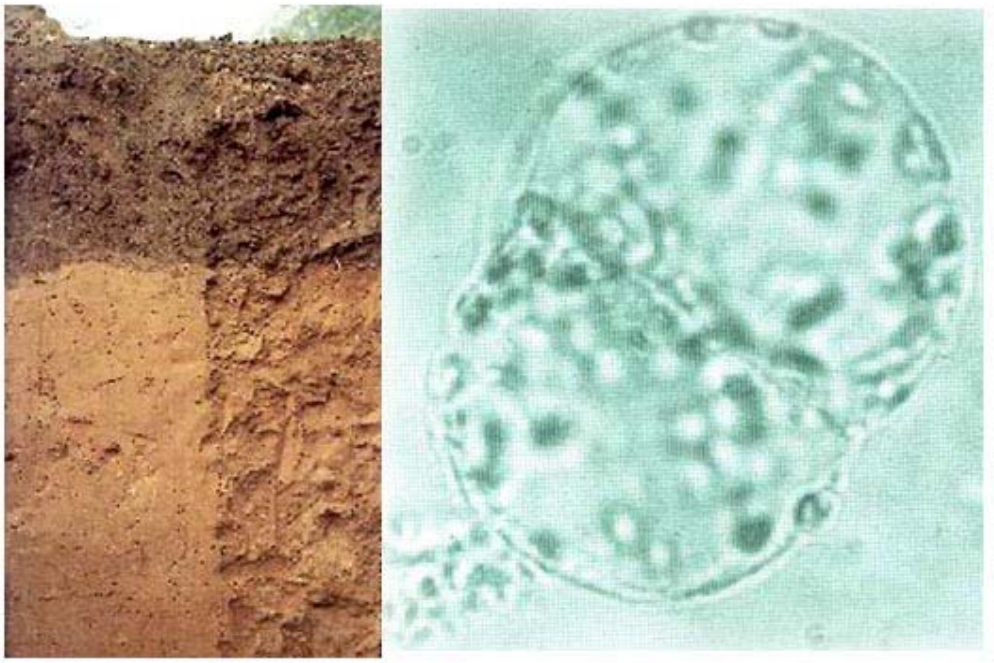
From soil to cell – a broad approach to plant life



Edited by L. Bender and A. Kumar

Giessen 2001

Dedicated to

Prof. Dr. Karl-Hermann Neumann

at the occassion of his retirement

Editors:

Prof. Dr. Ludwig Bender, Faculty of Agricultural Sciences, Department of Horticulture,
University of Applied Sciences Osnabrück, Oldenburger Landstrasse 24,
D - 49090 Osnabrück; e-mail:mailto:l.bender@fh-osnabrueck.de

Prof. Dr. Ashwani Kumar, Department of Botany, University of Rajasthan,
Jaipur, India; e-mail: msku4@hotmail.com

Preface

In the early 70th Professor Neumann had established a plant cell and tissue culture laboratory at the Institute of Plant Nutrition of the Justus Liebig-University Giessen, Germany and brought forward the idea to study plant developmental and nutritional phenomena involved in growth and yield formation by employing cell and tissue culture techniques, which was a novel approach for research in plant nutritional at that time.

Professor Neumann's thinking was certainly inspired by his academic mentors H. Linser from Giessen who pursued the concept of System and Product Growth in plant development and yield formation, and F. C. Steward, Cornell University, Ithaca, NY, who was among the first to employ plant cell and tissue culture techniques for studying hormonal and metabolic regulation of cell differentiation and plant development.

Consequently, over the next few decades, Professor Neumann and his team have been working on plant cell differentiation and growth regulation at the molecular, genomic, cytological and morphological levels. In vitro and in vivo studies were carried out on the effect of hormonal, nutritional and physical factors influencing the formation of organs and somatic embryos, DNA amplification and gene expression. The development of chloroplasts and the function of the photosynthetic system in cultured cells as well as the production of secondary metabolites by fermenter grown cell cultures were also some of the aspects he and his team examined. Above all there was the ever intriguing question as to how development and yield formation in higher plants is brought about. In recent years he could witness some of his early ideas of employing molecular methodologies in studies on plant nutrition coming to fruition [see Arnholdt-Schmitt (1996): Basic Strategies of Molecular Biological Research in Plant Nutrition - a Review. J. Plant Nutrition and Soil Sciences 159, 317-326] .
Besides the work related to cell biology, soil oriented studies were the other major topic for Professor Neumann and some of his team. For at least two decades, he was involved in research on soil physics and chemistry as influenced by irrigation and salinity conducted in Germany (the so called "Ried Project"), Egypt and India. It is certainly for that reason that the present volume honoring his scientific work is entiteled "From Soil to Cell - a Broad Approach to Plant Life", and that it covers topics ranging from soil-plant interrelations through plant cell and tissue culture techniques to biochemical and molecular genetic aspects.

Furthermore, what made working with Karl-Hermann Neumann and in his team a most rewarding and memorable experience for his students and collaborators was not only his scientific focus and insight, but also his personality - hard working at the research and fund acquisition front to keep us all going, yet always friendly and fair with more than one open ear for academic or personal matters at any time. We are all deeply indebted to him.

Osnabrück, Summer 2001 Ludwig Bender Ashwani Kumar

Content

Biodiversity of the Indian Desert and it´s Value

Amit Kotia and Ashwani Kumar

Biotechnology Lab, Department of Botany
University of Rajasthan, Jaipur – 302004 INDIA

Abstract

The state of Rajasthan is situated between 23º3' and 30º12' N latitude and 69º30' and 78º17' E longitude . The total land area of the state is about 3,24,239 km² , out of which about 1,98,100 km² is arid and the rest semi arid. The physical features are characterized mainly by the Aravallis and to the some extent by the vindhyan formation, and the Deccan trap. A major portion of western Rajasthan has desert soils and sandy plains. Sand dunes occupy a greater part of western Rajasthan (1,20, 983 km²). The soils of the desert plains are loamy sand to loam and the eastern part has alluvial soil which supports good forests and agricultural crop. Occurrence of saline soils with pH up to 9.0 is a common feature in the sandy areas of Rajasthan. The average annual rainfall in the state is 525-675 mm, and the annual precipitation in different tracts of Rajasthan varies from 13 mm to 1766 mm. Out of the total area , forests cover only about 37,638 km² and are rich in biodiversity. Rajasthan is rich in biodiversity which has a great economic value. Characterization of different plant species of economic value was undertaken. (Table 1-6).

INTRODUCTION

Out of the total land area of Rajasthan , forest covers only about 37,638 km², i.e. 11 %. This forest includes roughly 7 % of depleted and denuded forests. Biodiversity of Rajasthan is related with the Aravalli hills. *Anogeissus pendula* Edgew. forests cover more than half of the total forest area in the state. These forests occur on a variety of rock formations on the Aravalli hills. *A. pendula* Edgew. is also found in the southern region of the Vindhyan formations. It is able to grow on stony, impoverished and shallow soils, and also on a range of sandy loams to clay loam. *A. pendula* Edgew forms pure stands. It is commonly associated with *Diospyros melanoxylon* Roxb., *Acacia leucophloea* Willd., *Bauhinia racemosa* Lam. And *Wrightia tinctoria* R. Br. In parts of the Jaipur, Ajmer and Jodhpur districts *Acacia Senegal* Willd. is common. On the upper slopes , the main species are replaced by *Sterculia urens* Roxb., *Boswellia serrata* Roxb. and *Lannea coromandelica* (Houtt.) Herrill. and along the foothills by *Butea monosperma* (Lam.) Taub. The other species found are *Dichrostachys cinerea* (L.) Wt. & Arn., *Balanites aegyptiaca* (L.) Del., *Maytens emerginata* (Willd.) D. Hou, *Rhus mysurensis* Heyne, *Securinga leucopyrus* (Willd.) Muell. Arg., *Grewia flavescens* Jurs., *G. tenas* (Forsk.) Fiori and *Lycium barbarum* Linn. (Roy and Kumar , 1987). *Acacia catechu* Willd. forests are common in the south-eastern regions. e.g. Baran, Jhalawar, Kota, Tonk , Chittorgarh and Alwar. The area under this type is roughly 3 % of the total forest area. (Roy and Kumar , 1995; Kumar and Roy 1996).

The Rajasthan desert has extensive areas of saline soil which can be effectively utilized for biomass production. Notable among the species, including halopytes, which can be raised in this area include *Tamarix troupii* Hole, *Acacia nilotica* (L.) Del., *Calotropis* procera (Ait.) R.Br., *Capparis deciduas* (Forsk.) Edgew, *Salvadora oleoides* Decne., *Prosopis chilensis*

(Molina) Stuntz. These can be raised on soils with lower levels of salinity. Some of the grasses which can be grown are *Aeluropus logopoides* (L.) Trin. ex Thw., *Dactyloctenium aegyptium* (L.) P. Beauv., *Eleusine compressa* (Forsk.) Ascheros. and Schweing and *Eragrostis ciliaris* (L.) R.Br. (Kumar , 1987.)
The vast sandy tracts which are distributed in the Western and Northern plains of the state, form the dunes and the plain. The dunes are of two type - the embryonic, and the stabilized ones. There is no vegetation on the embryonic dunes except some ephemerals like *Gisekia pharnaceoides, Euphorbia prostrata, Mollugo cerviana, Polycarpaea corymbosa* and others which are the pioneers. When the embryonic dunes are gradually stabilized due to the growth of sand binders like *Leptadenia pyrotechnica, Calotropis procera, Aerva tomentosa, Saccharam munja,* and others , they provide a suitable habitat for the growth of some annual grasses, e.g. species of *Eragostis, Aristida*, etc. Plant species like *Convolvulus, Heliotropium, Indigofera, Tephrosia, Polygala,* and perennials like *Echinops echinatus, Crotalaria medicagenia* , and Shrub like *Acacia jacqumontii.* Besides this *Trianthema, Chenopodium, Salsol* and *Suaeda* plants are very common in saline regions of Rajasthan.

Material and Methods

The plant species were collected from different sites of the state like the Ajmer, Churu, Jhunjhunu, Dausa, Sikar, and mainly the Jaipur district. Identification of these plant species was done using standard monographs and their local flora (Sharma , 1976; Bhandari, 1978). The survey of these sites was carried out over a period of two years (1999-2000). The plant specimens have been deposited in the herbarium of the Botany Department, University of Rajasthan, Jaipur.

Observation

The following plant species have been recorded from different area of the state in different seasons. This plant biodiversity has lots of economic value. Some of the plants are listed in the following table. They include different categories like plants yielding Fibers, Tannins, Dyes, Gum and Resins, Extraction & Distillation products, plants for lac worm hosts, Plant for Silkworm hosts, Biri leaves, Soap Substitutes etc.

A. Trees:

Acacia leucophloea (Roxb.) Willd. ; *Acacia nilotica* (Linn.) Willd. ex Del. ; *Acacia senegal* (Linn.) Willd. ; *Acacia tortilis (*Forsk.*)* Hayne.*(Figure 1); Azadirachta indica* A. Juss.; *Ailanthus excelsa* Roxb.; *Balanites aegyptiaca* (Linn.) Delile.; *Dichrostachys cinerea* (Linn.) Wight. et Arn.; *Ficus benghalensis* Linn.; *Ficus religiosa* Linn.; *Holoptelea integrifolia* Planch.; *Prosopis cineraria* (Linn.) Druce.; *Prosopis juliflora* (Swartz.) DC. ; *Tecomella undulata* (Sm.) Seeman..*; Ziziphus mauritiana* Lamk.; *Maytenus emarginata* (Willd.) Ding-Hou. *Phoenix sylvestris* (Linn.) Roxb.; *Riccinus communis* Linn.; *Terminalia alata* Heyne *; Terminalia arjuna (R*oxb.) Wight. & Ara.*; Cassia fistula* Linn.*; Cassia auriculata* Linn; . *Tamarix aphylla* (L.) Karst.; *Pithecellobium dulce* Benth.; *Acacia catechu* Willd.*; Zizyphus glaberrima* Santapau.; *Terminalia bellirica* Roxb.*; Emblica officinalis* Gaertn.*; Anogeissus pendula* Edgew.*; Anogeissus latifolia* Wall.; *Prosopis cineraria* (L.) Druce.; *Garuga pinnata* Roxb.*;*

Madhuca indica Gmel.*; Pongamia pinnata* (L.) Pierre.*; Salvadora oleoides* Decne.; *Salvadora persica* Linn.*; Jatropha curcas* Linn.; *Balanites aegyptiaca* (L.) Delile.; *Sapindus emarginatus* Vahl.*; Mimusops elengi* Linn.*; Aegle marmelos* (L.) Correa.; *Bauhinia racemosa* Lamk.; *Boswellia serrata* Roxb.*; Bombax ceiba* Linn.*; Buchnania latifolia* Roxb.*; Butea monosperma* (Lamk.) Taub.; Leucaena leucocephala (Lam.) de Wit.; *Lannea coromandelica* (Houtt.) Merril.; *Moringa oleifera* Lam.*; Mangifera indica* Linn.*; Miliusa tomentosa* (Roxb.) J. Sinclair; *Pterocarpus marsupium* Roxb.*; Sterculia urens* Roxb.; *Nyctanthes arbortristis* Linn.; *Wrightia tinctoria* R.Br.*; Morinda tinctoria* Roxb.*; Helicteres isora* Linn.*; Cordia gharf* (Farsk.) Her. & Asch.; *Erythrina suberosa*
Roxb.*; Phoenix sylvestre* Roxb.*; Cordia oblique* Willd. *Ficus religiosa* Linn.*; Morus alba* Linn.*; Diospryos melanoxylon* Roxb.*; Diospryos tomentosa* Roxb.*; Diospryos montana* Roxb.*; Santalum album* Linn.

B. Shrubs:

Carissa carandas Linn; *Punica granatum* Linn.*; Lawsonia inermis* Linn.*;Rhus mysurensis* Heyne.; *Mallotus philiphinensis* Muell. – Arg.*; Capparis deciduas* (Forsk.) Edgew.; *Abutilon indicum* (Linn.) Sweet.; *Sida cordifolia* Linn. ; *Waltheria indica* Linn.; *Commiphara wightii* (Arn.) Bhandari.; *Hibiscus ovalifolius* Vahl.; *Ziziphus nummularia* (Burm.f.) Wt.et Arn.; *Acacia jacqumontii* Benth.; *Crotalaria burhia* Buch.-Ham. ex Benth.; *Grewia tenax* (Forsk.) Fiori.; *Crotalaria medicaginia* Lamk.; *Verbesina encelioides* (Cav.) Benth. & Hook.; *Xanthium strumarium* Linn.; *Calotropis procera* (Ait.) R.Br.; *Leptadenia pyrotechnica* (Forsk.) Decne.; *Sericostoma pauciflorum* Stocks.; *Withania somnifera* (Linn.) Dunal.; *Lantana indica* Roxb.; *Aerva tomentosa* (Burm. f.) Juss.; *Salsola baryosoma* (R.et S.) Dandy.; *Suaeda maritima* (Linn.) Dumort.

C. Perennial herbs :

Tephrosia hamiltonii Drumm.; *Tephrosia purpurea* (Linn.) Pers.; *Farsetia hamiltonii* Royle.; *Indigofera linnaei* Ali.; *Trianthema portulacastrum* Linn.; *Zaleya govindia* (Buch-Ham. ex G. Don) N.C. Nair.; *Borreria articularis* (Linn.) F.N. Will..; *Echinops echinatus* Roxb.; *Launaea resedifolia* (Linn.) Druce.*; Launaea procumbens* (Roxb.) Rammyya et Rajgopal.; *Oligochaeta ramose* (Roxb.) Wagenitz.; *Pulicaria crispa* Sch.-Bip.; *Catharanthus roseus* (Linn.) Don.; *Convolvulus microphyllous* Sieb. ex Spreng.; *Datura metal* Linn. ; *Solanum nigrum* Linn.; *Solanum surattense* Burm.; *Lepidagathis trinervis* Wall. ex Nees.(Figure, 2); *Boerhavia diffusa* Linn.; *Achyranthes aspera* Linn.; *Amaranthus caudatus* Linn.; *Pupalia lappacea* (Linn.) Juss.; *Croton bonplandianum* Baill.; *Euphorbia hirta* Linn.

D. Annual herbs :

Argemone mexicana Linn.; *Fumaria indica* (Haussk.) Pugsley.; *Sismbrium irio* Linn.; *Portulaca oleracea* Linn.; *Portulaca suffruticosa* Wt.; *Alysicarpus monilifer* DC.; *Medicago laciniata* (Linn.) Mill., *Melilotus indica* All.; *Fagonia cretica* Linn.; *Trigonella. polycerata* Linn.; *Trianthema triquetra* Rottl. ex Willd.; *Acanthospermum hispidum* DC. (Figure 4); *Artemisia scoparia* Waldst et Kit.; *Gnaphalium indicum* Linn.; *Pulicaria angustifolia* DC.; *Sonchus asper* (Linn.) Gars.; *Vernonia cinerea* (Linn.) Less.; *Anagallis arvensis* Linn.; *Arnebia hispidissima* (Lehm.) DC.; *Heliotropium ellipticum* Ledeb. (Figure 3); *Heliotropium marifolium Retz.; Heliotropium subulatum* Hochst. ex
DC.; *Datura innoxia* Mill.; *Leucas aspera* (Willd.) Spreng.; *Gomphrena celosiodies* Mart.*; Indigofera cordifolia* Heyne.; *Indigofera hochstetteri* Baker.; *Tephrosia*

strigosa (Dalz.) Sant.; *Ocimum canum* Sims.; *Chenopodium album* Linn.; *Chenopodium murale* Linn.; *Phyllanthus asperulatus* Hutch.

E. Ephemerals :

Cleome gynandra Linn.; *Cleome viscose* Linn.; *Polygala erioptera* DC.; *Polygala irregularis* Boiss.; *Indigofera astragalina* DC.; *Polycarpaea corymbosa* (Linn.) Lamk.; *Sida ovata* Forst.; *Corchorus tridens* Linn.; *Triumfetta pentandra* A.Rich.; *Tribulus terrestris* Linn.; *Cassia tora* Linn. (Figure 6); *Cassia occidentalis* Linn.; *Alysicarpus vaginalis* (Linn.) DC.; *Indigofera linifolia* (Linn.) Retz.; *Indigofera sessiliflora* DC.; *Gisekia pharnaceoides* Linn.; *Mollugo cerviana* (Linn.) Ser. (Figure 7); *Mollugo nudicaulis* Lamk.; *Bidens biternata* (Lour.) Merr. & Sherff.; *Blainvillea acmella* (Linn.) Philipson.; *Trichodesma indicum* R.Br.; *Evolvulus alsinoides* Linn.; *Physalis minima* Linn.; *Pedalium murex* Linn.; *Sesamum indicum* Linn.; *Martynia annua* Linn.; *Peristrophe bicalyculata* (Retz.) Nees.; *Rostellularia procumbens* (Linn.) Ness.; *Anisomeles indica* (Linn.) Ktze. (Figure 5); *Amaranthus spinosus* Linn.; *Digera muricata* (Linn.) Mart.; *Euphorbia prostrata* Ait.; *Commelina benghalensis* Linn.; *Commelina forskalaei* Vahl.

F. Climbers and twinners:

Cocculus pendulus (Forst.) Diels.; *Celastrus paniculata* Willd.; *Tinospora cordifolia* (Willd.) Miers.; *Blastania fimbristipula* (Fensl.) Kotschy et Peyr.; *Citrullus colocynthis* (Linn.) Schrad.(Figure 8); *Cucumis callosus* (Rottl.) Cogn.; *Mukia maderaspatana* (Linn.) M. Roem.; *Pergularia daemia* (Forsk.) Chiov.; *Ipomoea eriocarpa* R.Br.; *Ipomoea pes-tigridis* Linn.

G. Grasses :

Bulbostylis barbata (Rottb.) Kunth.; *Cyperus arenarius Retz.; Cyperus bulbosus* Vahl.; *Cyperus triceps* (Rottb.) Endl.; *Aristida funiculata* Trin. et Rupr.; *Brachiaria ramose* (Linn.) Stapf .; *Brachiaria reptans* (Linn.) Gardener et Hubb.; *Cenchrus biflorus* Roxb.; *Cenchrus ciliaris* Linn.(Figure, 9); *Cenchrus pennisetiformis* Hochst. et Steud.; *Chloris virgata* Sw.; *Dactyloctenium sindicum* Boiss.; *Vetiveria zizanioides* (L.) Nash.; *Typha elephantine* Roxb.; *Eragrostis ciliaris* (Linn.) R.Br.; *Eragrostis pilosa* (Linn.) P. Beauv.; *Eragrostis tremula* Hochst. ex Steud.; *Saccharum bengalense* Retz.

Conclusion:

This report based on the survey of plant biodiversity of different sites over a period of 2 ½ years. Seasonal appearances at the sites of study were recorded (Kotia and Kumar , 2001a,c). Two hundred twelve species were listed, belonging to one hundred ten genera of flowering plants. About 50 species carry medicinal importance as can be derived from Ayurvedic literature (Jain, 1968; Jain, 1991; Kotia and Kumar, 2001b).Besides this, some of them are used as fodders and others provide edible fruits.

The rich biodiversity of Rajasthan has a great potential of plants having medicinal value. In addition to this, these plants provide tannins, oils, gums and resins, dyes, and fibers. However, their characterization is lacking. The present paper has attempted to record the data regarding the availability of plant resources which have various potential uses. The detailed investigation on the molecular and genetic characterization of these plants is necessary to have gene pool conservation. Attempts are also under way to make an in situ gene pool bank for the plants for further researches.

Table 1: Tannins yielding plants of Rajasthan

Local Name	Botanical Name	Family	Part use	Other uses
Babul	*Acacia nilotica* (L.) Willd.	Mimosaceae	Bark	Medicinally, Gum
Arunj	*Acacia leucophloea* Willd.	Mimosaceae	Bark	Fiber , Gum
Sadad	*Terminalia alata* Heyne.	Combretaceae	Bark	Medicinally
Arjuna	*Terminalia arjuna (R*oxb.) Wight. & Ara.	Combretaceae	Bark	Medicinally, silk worm host
Amaltas	*Cassia fistula* Linn.	Caesalpiniaceae	Bark	Medicinally
Anwal	*Cassia auriculata* Linn.	Caesalpiniaceae	Bark	Medicinally
Godal	*Lannea coromandelica* (Houtt.) Merrill.	Anacardiaceae	Bark	Gum, Resin, Dye
Dansara	*Rhus mysurensis* Heyne.	Anacardiaceae	Bark	Fruits edible
Farash	*Tamarix aphylla* (L.) Karst.	Tamaricaceae	Bark	Medicinally
Jangaljalbi	*Pithecellobium dulce* Benth.	Mimosaceae	Bark	Timber
Khair	*Acacia catechu* Willd.	Mimosaceae	Bark	Dye, Medicinally, Gum
Ghatbor	*Zizyphus glaberrima* Santapau.	Rhamnaceae	Fruit	Fruit edible
Baheda	*Terminalia bellirica* Roxb.	Combretaceae	Fruit	Medicinally
Anonla	*Emblica officinalis* Gaertn.	Euphorbiaceae	Fruit	Fruit edible , Medicinally
Dhokra	*Anogeissus pendula* Edgew.	Combretaceae	Leaves	Medicinally
Dhawra	*Anogeissus latifolia* Wall.	Combretaceae	Leaves	Gum, Medicinally

Karaunda	*Carissa carandas* Linn.	Apocynaceae	Leaves	Fruit edible
Mehandi	*Lawsonia inermis* Linn.	Lytharaceae	Leaves	Dye, Medicinally
Khejari	*Prosopis cineraria* (L.) Druce.	Mimosaceae	Leaves	Fruit edible, Gums
Karpata	*Garuga pinnata* Roxb.	Burseraceae	Leaves	Medicinally

Table 2 : Non edible oil yielding plants of Rajasthan.

Local Name	Botanical Name	Family	Part use	Other uses
Arundi	*Riccinus communis* Linn.	Euphorbiacae	Seed	Medicinally
Mahuwa	*Madhuca indica* Gmel.	Sapotaceae	Seed	Fruit edible
Karanj	*Pongamia pinnata* (L.) Pierre.	Fabaceae	Seed	Medicinally
Neem	*Azadiractha indica* A.Juss.	Meliaceae	Seed	Medicinally
Tumba	*Citrullus colocynthis* (L.) Schard.	Cucurbitaceae	Seed	Medicinally
Pili-hulhul	*Cleome viscose* Linn.	Capparaceae	Seed	Medicinally
Satyanashi	*Argemone maxicana* Linn.	Papaveraceae	Seed	Medicinally
Pilu	*Salvadora oleoides* Decne.	Salvadoraceae	Seed	Medicinally
Kharajal	*Salvadora persica* Linn.	Salvadoraceae	Seed	Medicinally
Ratanjot	*Jatropha curcas* Linn.	Euphorbiaceae	Seed	Medicinally, Dye
Hingot	*Balanites aegyptiaca* (L.) Delile.	Simaroubaceae	Seed	Medicinally
Aritha	*Sapindus emarginatus* Vahl.	Sapindaceae	Seed	Soap Substitutes
Maulsiri	*Mimusops elengi* Linn.	Sapotaceae	Seed	Medicinally
Malkangini	*Celastrus paniculata* Willd.	Celastraceae	Seed	Medicinally

Table 3 : Gums & Resins yielding plants of Rajasthan.

Local Name	Botanical Name	Family	Other uses
Babul	*Acacia nilotica* (L.) Willd .	Mimosaceae	Tannin, Medicinally
Kumta	*Acacia senegal* Willd.	Mimosaceae	Tannin, Medicinally
Baonli	*Acacia jacquemontii* Benth..	Mimosaceae	Medicinally
Arunj	*Acacia leucophloea* (Roxb.) Willd.	Mimosaceae	Medicinally , Tannin
Dhawra	*Anogeissus latifolia* Wall.	Combretaceae	Taninn, Medicinally
Dhokra	*Anogeissus pendula* Edgew.	Combretaceae	Medicinally
Beel	*Aegle marmelos* (L.) Correa.	Rutaceae	Medicinally, Fruit edible
Neem	*Azadirachta indica* A.Juss.	Meliaceae	Oil, Medicinally
Jhinjha	*Bauhinia racemosa* Lamk.	Caesalpiniaceae	Medicinally
Salar	*Boswellia serrata* Roxb.	Burse raceae	Medicinally
Semal	*Bombax ceiba* Linn.	Bombacaceae	Fiber
Chironji	*Buchnania latifolia* Roxb.	Anacardiaceae	Seed edible
Palas	*Butea monosperma* (Lamk.) Taub.	Fabaceae	Dye, Medicinally
Ganiara	*Cochlospermum religiosum* (L.) Alston.	Cochlospermaceae	Fiber, Oil
Gugal	*Commiphara wightii* (Arn.) Bhandari	Burseraceae	Medicinally
Subabool	Leucaena leucocephala (Lam.) de Wit.	Mimosaceae	Firewood, Charcoal
Godal	*Lannea coromandelica* (Houtt.) Merril.	Anacardiaceae	Dye, Timber, Tannin
Sainjana	*Moringa oleifera* Lam.	Caesalpiniaceae	Fruit edible
Aam	*Mangifera indica* Linn.	Anacardiaceae	Fruit edible
Umb	*Miliusa tomentosa* (Roxb.) J. Sinclair	Anonaceae	Timber
Bijasal	*Pterocarpus marsupium* Roxb.	Fabaceae	Medicinally
Katria	*Sterculia urens* Roxb.	Sterculiaceae	Oil, Medicinally
Rohan	*Soymida febrifuga* A. Juss.	Meliaceae	Medicinally
Khair	*Acacia catechu* Willd	Mimosaceae	Dye , Medicinally , Tannin

Table 4 : Dyes yielding plants of Rajasthan

Local Name	Botanical Name	Family	Part use	Other uses
Khair	*Acacia catechu* Willd.	Mimosaceae	Wood	Tannin, Medicinally
Sadad	*Terminalia alata* Heyne.	Combertaceae	Bark	Medicinally
Maulsiri	*Mimusops elengi* Linn.	Sapotaceae	Bark	Fruit edible
Godal	*Lannea coromandelica (*Houtt.*)* Merrill.	Anacardiaceae	Bark	Dye, Gum, Resine
Kamala	*Mallotus philiphinensis* Muell. – Arg.	Euphorbiaceae	Fruit	Non edible oil
Palas	*Butea monosperma* (Lamk.) Taub.	Fabaceae	Flower	Medicinally, Gum
Harsinghar	*Nyctanthes arbortristis* Linn.	Oleaceae	Flower	Essential oil
Khirni	*Wrightia tinctoria* R.Br.	Apocynaceae	Flower	Medicinally
Aal	*Morinda tinctoria* Roxb.	Rubiaceae	Root	Medicinally
Anar	*Punica granatum* Linn.	Punicaceae	Root	Fruit edible
Mehandi	*Lawsonia inermis* Linn.	Lytharaceae	Leaves	Medicinally

Table 5 : Fibers yielding plants of Rajasthan.

Local Name	Botanical Name	Family	Part use	Other uses
Palas	*Butea monosperma* (Lamk.) Taub.	Fabaceae	Bark	Dye, Medicinally
Karaya	*Sterculia urens* Roxb.	Sterculiaceae	Bark	Gum, Resin,
Mororphali	*Helicteres isora* Linn.	Sterculiaceae	Bark	Medicinally
Sandan	*Ougeinia oojeinesis* (Roxb.) Hochreut	Fabaceae	Bark	Poisonous plant
Kewra	*Pandanus tectorius* Sol. ex. Rark.	Pandanaceae	Leaves	Medicinally
Aira	*Typha elephantina* Roxb.	Typhaceae	Leaves	Wasteland colonizer
Jhinjha	*Bauhinia racemosa* Lamk.	Caesalpiniaceae	Bark	Medicinally
Aak	*Calotropis procera* (Ait.) R.Br.	Asclepiadaceae	Bark	Medicinally
Semal	*Bombax ceiba* Linn.	Bombacaceae	Fruit & Flower	Ornamental Plant
Arunj	*Acacia leucophloea (*Roxb.*)* Willd.	Mimosaceae	Bark	Tannins , Gum, Resin
Gondi	*Cordia gharf* (Farsk.) Her. & Asch.	Ehretiaceae	Bark	Medicinally
Gadha Palas	*Erythrina suberosa* Roxb.	Fabaceae	Bark	Medicinally
Gangan	*Grewia tenax (*Forsk.*)* Fiori.	Tiliaceae	Bark	Medicinally
Khimp	*Leptadenia pyrotechnica (*Forsk.*)* Decne.	Asclepiadaceae	Stem	Medicinally, Fruit edible
Khajur	*Phoenix sylvestre* Roxb.	Arecaceae	Leaves	Fruit edibal
Tad	*Borassus flabellifer* Linn.	Arecaceae	Fruit & Flower	Seed edible
Dudhi	*Wrightia tinctoria* R.Br.	Apocynaceae	Fruit & Flower	Medicinally
Ganiara	*Cochlospermum religiosum* (L.) Alston.	Cochlospermaceae	Fruit & Flower	Gum & Resin
Rambans	*Agave americana* Linn.	Agavaceae	Leaves	Medicinally
Senia	*Crotalaria burhia* Buch. Ham.	Fabaceae	Stem	Wasteland colonizer
Gonda	*Cordia oblique* Willd.	Ehretiaceae	Bark	Medicinally

Table 6 : Other Economically important plants of Rajasthan.

Local Name	Botanical Name	Family	Uses
Ber	*Zizyphus mauritiana* Lamk..	Rhamnaceae	Lakh worm host
Ghatbor	*Zizyphus glaberrima* Santapau.	Rhamnaceae	Lakh worm host
Palas	*Butea monosperma (*Lamk.*)* Taub.	Fabaceae	Lakh worm host
Pipal	*Ficus religiosa* Linn.	Moraceae	Lakh worm hosts
Shahtut	*Morus alba* Linn.	Moraceae	Silk worm host
Arjuna	*Terminalia arjuna (*Roxb.*)* Wight. & Arn.	Combretaceae	Silk worm host
Arundi	*Riccinus communis* Linn.	Euphorbiaceae	Silk worm host
Timru	*Diospryos melanoxylon* Roxb.	Ebenaceae	Biri leaves
Tendu	*Diospryos tomentosa* Roxb.	Ebenaceae	Biri leaves
Chikon	*Diospryos montana* Roxb.	Ebenaceae	Biri leaves
Jhinjha	*Bauhinia racemosa* Lamk.	Caesalpiniaceae	Biri leaves
Aritha	*Sapindus emarginatus* Vahl.	Sapindaceae	Soap substitutes
Hingot	*Balanites aegyptiaca* (L.) Delile.	Simaroubaceae	Soap substitutes
Khus	*Vetiveria zizanioides* (L.) Nash.	Poaceae	Extraction and Distilation products
Khair	*Acacia catechu* Willd..	Mimosaceae	Extraction and Distilation products
Mahuwa	*Madhuca indica* Gmel.	Sapotaceae	Extraction and Distilation products
Rosha	*Cymbopogon martini* (Roxb.) Wats.	Poaceae	Extraction and Distilation products
Chandan	*Santalum album* Linn.	Santalaceae	Extraction and Distilation products

Figure 1: *Acacia tortilis* (Forsk.) Hayne

Figure 2: *Lepidagathis trinervis* Wall. Ex Nees.

Figure 3: *Heliotropium ellipticum* Ledeb.

Figure 4: *Acanthosperum hispidum* DC

Figure 5: *Anisomeles indica* (Linn.) Ktze.

Figure 6: *Cassia tora* (Linn.)

Figure 7: *Mollugo cerviana* (Linn.) Ser.

Figure 8: *Citrullus colosynthis* (Linn.) Schrad

Figure 9: *Cenchrus ciliaris* (Linn.)

References

Bhandari, M.M. (1978): Flora Of Indian Desert, Scientific Publishers, Jodhpur, 1- 466.

Jain, S.K. (1968): Medicinal Plants, National Book Trust, New Delhi.

Jain, S.K. and R.A. Defellips (1991): In : Medicinal Plant Of India, Reference Publication, Algonal, Michigan, U.S.A.

Kotia, A. and A. Kumar (2001a): Characterization of weeds on Wastelands and their role in Eco-development. Int. J. Mendel, Vol 18 (1-2), Page 7-8.

Kotia, A. and A. Kumar (2001b): Some of the common weeds of medicinal value from Rajasthan. Int. J. Mendel, Vol 18 (1-2), Page 17-18.

Kotia, A. and A. Kumar (2001c): Characterization of biomass during wasteland development in semiarid region. J. of Environment and Pollution 8, 213-216.

Kumar, A. (1987): Petro Crop Resources of Rajasthan. In: Proceedings Bio-Energy Society, IVth Convention & Symposium, 87. (R.N. Sharma, O.P. Vimal and A.N. Mathur, eds.); Bio-Energy Society of India, New Delhi; pp. 106-111.

Kumar , A. and S. Roy (1996): Biomass resources of Semi-arid regions. Production and improvement of wood energy source. In: Biomass for energy and environment (P. Chartier, U.M. Henius, S.Hultberg, J. Sachau and M. Wiinblad, eds.) Elsevier science, U.K.; pp. 721-724.

Roy , S. and A. Kumar (1987): Potential of Different Tree species as sources of Biomass in Rajasthan. In: Proceedings Bio Energy Society IVth Convention & Symposium 87. (R.N. Sharma, O.P. Vimal and A.N. Mathur, eds.); Bio-Energy Society of India, New Delhi; pp. 62-66.

Roy , S. and A. Kumar (1995): Biodiversity of Rajasthan and its energy potentials.
J. Environment and Pollution 2, 105-109.

Sharma , S. (1976): Flora of North East Rajasthan , Kalyani Publisher. New Delhi; pp. 1-395.

Bioengineering of Crops for Biofuels and Bioenergy

Ashwani Kumar

Energy Plantation Demonstration Project Center and Biotechnology Laboratory,

Department of Botany, University of Rajasthan,

Jaipur, 302004. India.

e-mail. msku4@hotmail.com

Abstract

Biomass contributes a significant share of global primary energy consumption and its importance is likely to increase in future world energy scenarios. Current biomass use, although not sustainable in some cases, replaces fossil fuel consumption and results in avoided CO_2 emissions, representing about 2.7 to 8.8 % of 1998 anthropogenic CO_2 emissions. The global biomass energy potential is large, estimated at about 104 EJ/a. Hence, biomass has the potential to avoid significant fossil fuel consumption, potentially between 17 and 36 % of the current level and CO_2 emissions potentially between 12 and 44 % of the 1998 level. Modern biomass energy use can contribute to controlling CO_2 emissions to the atmosphere while fostering local and regional development. There is significant scope then to integrate biomass energy with agriculture, forestry and climate change policies.
Further the advantages from utilization of biomass include: liquid fuels produced from biomass contain no sulfur, thus avoiding SO_2 emissions and also reducing emission of $N0_x$. The production of compost as a soil conditioner avoids deterioration of soil. Improved agronomic practices of well managed biomass plantations will also provide a basis for environmental improvement by helping to stabilize certain soils, avoiding desertification which is already occurring rapidly in tropical countries. The creation of new employment opportunities within the community and particularly in rural areas will be one of the major social benefits. The specific research work carried out in the areas of biomass production and utilization in less fertile areas will provide satisfactory answers to the double challenge of energy crisis and forced deforestation in the country and semi-arid and arid regions of Rajasthan. The possibility of conversion of biomass into liquid fuels and electricity will make it possible to supply part of the increasing demand for primary energy and thus reduce crude petroleum imports which entail heavy expenditure on foreign exchange. The families Euphorbiaceae (*Euphorbia antisyphilitica, E.tithymaloides, E. caducifolia E. royleana E. neerifolia* etc. and Ascelpiadaceae (*Calotropis gigantea* and *C. procera*) which have been worked out in previous years (Kumar, 2000) will form the basis for further studies.

Introduction

Worldwide energy consumption is projected to grow by 59 % over the next two decades, according to International Energy Outlook 2001 (IEO 2001), released by theUS Energy

Information Administration (EIA). One half of the projected growth is expected to occur in the developing nations of Asia (including China, India and South Korea) and in Central and South America, where strong economic growth is likely to spur demand for energy over the forecast period. Renewable energy use is expected to increase by 53 % between 1999 and 2020, but its current 9 % share of total energy consumption is projected to drop to 8 % by 2020. Oil currently accounts for a larger share of world energy consumption than any other energy source and is expected to remain in that position throughout the forecast period. World oil use is projected to increase from 75 million barrels per day in 1999 to 120 million barrels per day in 2020.

Biomass resources are potentially the worlds largest renewable energy source – at an annual terrestrial biomass yield of 220 billion oven dry tonnes. Biomass conversion to fuel and chemicals is once again becoming an important alternative to replace oil and coal. Biodiesel from the rape seed oil methylester (RME) produced by farmer cooperatives is about 2000 t RME per year. A large facility of 15000 t RME per year is located at the oil mill at Bruck/Leitha in Austria. RME is excellent substitute for diesel. Already, European countries, mainly France, Italy, Germany and Austria are leading in biodiesel production, nearing 500,000 tons in 1997 out of which 250,000 was produced in France.(Statt, 1998) The production capacity of biodiesel in Germany was fully utilized in1997, the sold quantity amounting to roughly 100,000 t (Groenen,1998). The technologies for producing bio-oil are evolving rapidly with improving process performance , larger yield and better quality products. The challenge is to develop a process technology which can cope with the significant variation in the composition of the raw material. Another line of action is *Camelina sativa* . This plant was a traditional oilseed in Europe. It is considered a “low input high yield” plant which could enhance the environmental aspect of biodiesel. However, it has a higher Iodine number (160).

Carbon dioxide emission is projected to grow from 5.8 billion tonnes carbon equivalent in 1990 to 7.8 billion tonnes in 2010 and 9.8 billion tonnes by 2020. The Kyoto conference agreement last year is not far reaching but indicates the role clean energy sources will play in the future. Biomass is renewable, non pollutant and available world wide as agricultural residues, short rotation forests and crops . Thermochemical conversion using low temperature processes are among the suitable technologies to promote a sustainable and environmentally friendly development. Biomass can play a dual role in greenhouse gas mitigation related to the objectives of the United Nations Framework Convention on Climate Change (UNFCC) i.e. as an energy source to substitute for fossil fuels and as a carbon store.

The sustainable development of large areas of the world is today one of the greatest challenges . How will it be possible to provide the means for improving the socio-economic conditions of the increasing population in developing countries, a large part of which lives in villages and rural areas of Asia, Africa and South America. Biomass currently supplies about a third of the developing countries energy varying from about 90 % in countries like Uganda, Rwanda and Tanzania to 45 % in India, 30 % in China and Brazil and 10-15 % in Mexico and South Africa. Tropical deforestation is currently a significant environmental and development issue. The annual tropical deforestation rate for the decade 1981-1990 was about 15.4 million ha (Mha). According to some estimates the forest cover is 64.01 Mha accounting for 19.5 % of India’s geographic area. At present there is hardly 0.4 % forest cover below the 25 cm rainfall zone and 1.3 % above 30 cm. Since the annual photosynthetic production of biomass is about eight times the worlds total energy use and this energy can be produced and used in an environmentally suitable manner and mitigating net CO_2 emission, there can be little doubt that the potential source of stored energy must be carefully considered for future energy

needs. The fact that nearly 90 percent of the worlds population will reside in developing countries by about 2050 probably implies that biomass energy will be with us forever unless there are drastic changes in the world energy trading pattern.

Biomass should be used instead of fossil energy carriers in order to reduce i) CO_2 emissions ii) the anticipated resource scarcity of fossil fuels and iii) need to import fuels from abroad.

Current commercial and non-commercial biomass use for energy is estimated at between 20 and 60 EJ/a representing about 6 to 17 % of the world primary energy. Most of the biomass is used in developing countries where it is likely to account for roughly one third of primary energy. As a comparison, the share of primary energy provided by biomass in industrialized countries is small and is estimated at about 3 % or less.

Global land availability estimates for energy crop production vary widely between 350 and 950 million hectares (Alexandratos , 1995). An energy potential of about 37.4 EJ/a is estimate based on country specific biomass yield and an average land availability The worldwide technical biomass energy potential is then estimated at about 104 EJ/a corresponding to approximately one third of the global 320 EJ/a primary energy consumption of oil, gas and coal (BP-Amoco 1999).

The bio-oil consortium of the UK received huge grants (1.16 million pounds) to enable the commercial production and testing of an integrated bio-oil and electricity generating plant. UK´s energy minister Peter Hain ascribed " high priority to research and development of sustainable energy sources ". Commercial processing plants for the medium scale production of biodiesel from inter-esterification of triglycerides have been developed in France, Germany (CARMEN), Austria (ENERGIA Biodiesel Technology) USA (Ensyn Group Inc.) and in the EU (Eubia).
Liquid and gaseous transport fuels derived from a range of biomass sources are technically feasible. They include methanol, ethanol, dimethyl esters, pyrolytic oil, Fischer-Tropsch gasoline and distillate and biodiesel from (i) Jatropha , Pongamia pinnata, Salvadora persica, Madhuca longifolia and (ii) hydrocarbon from Euphorbia species.
Biomass energy is experiencing a surge in interest in many parts of the world due to a greater recognition of its current role and future potential contribution as modern fuel in the world energy supply, its availability, versatility and sustainable nature; a better understanding of its global and local environmental benefits, perceived potential role in climate stabilization, the existing and potential development and entrepreneurial opportunities. Technological advances and knowledge which have recently accumulated on many aspects of biomass energy, e.g. greater understanding of the possible conflict of food versus fuel etc. A recent World Bank report concluded that "Energy policies will need to be as concerned about the supply and use of biofuels as they are about modern fuels. (and) they must support ways to use bio-fuels more efficiently and in sustainable manner (World Bank, 1996)
Biomass resources are potentially the worlds largest and sustainable energy source a renewable resource comprising 220 billion oven dry tones (about 4500 EJ) of annual primary production. The annual bio-energy potential is about 2900 EJ though only 270 EJ could be considered available on a sustainable basis and at competitive prices.
Most major energy scenarios recognize bio-energy as an important component in the future worlds energy. Projections indicate the biomass energy use to the range of 85 EJ to 215 EJ in 2025 compared to the current global energy use of about 400 EJ of which 55 EJ are derived from biomass (Hall and Rosillo-Calle. 1998).

Despite the fact that biomass represents about one third of the energy consumption in developing countries, it is not taken very well into account in energy studies. A set of factors explain the slow growth on the biomass utilization . They include:

1. High costs of production
2. Limited potential for production
3. Lack of sufficient data on energy transformations coefficients.
4. Low energy efficiency
5. Health hazard in producing and using biomass.

In the large scale use of biomass for energy risks are insecurity in raw material supply and prices, doubts about adequate quality assurance and hesitance for a wider acceptance by the diesel engine manufacturers, missing marketing strategies for targeting biodiesel differential advantages into specific market niches and last not least missing legal frame conditions similar to the clean air act in the USA.

Energy Plantation Demonstration Project Center

Development of agrotechnolgies

The work on the development of suitable agro-technology for hydrocarbon yielding plants was initiated at the University of Rajasthan, Jaipur in 1980 with seeds of *Euphorbia lathyris* provided by Professor Melvin Calvin (Kumar, 1984). DST (Later on DNES) granted a research project to the principal investigator at the University of Rajasthan in 1982 to work on hydrocarbon yielding plants which was later raised to practical demonstration on 5 ha in 1985 after successful completion of first phase. This area was totally barren with only one tree as seen in the Figure 1 and 2.
After the successful demonstration of the second phase a project called Energy Plantation Demonstration Project for 50 has was granted in which a three tier system was followed as per the details of the work given below. A brief summary of the work done and important achievements is given below.

A 50 ha Energy Plantation Demonstration Project Center (EPDPC) in the semi- arid region of Rajasthan was used to conduct the investigations.
A large number of hydrocarbon yielding plants are able to grow under semi arid and arid conditions and they also produce valuable hydrocarbons (up to 30 % of dry matter) which could be converted into petroleum like substances and used as fossil fuel substitute. During the last 18 years investigations have been carried out on the optimization of yield and production of hydrocarbons by such plants at the 50 ha EPDPC of the University of Rajasthan, Jaipur. Their yield could be increased several fold making their commercial cultivation feasible.

Hydrocarbons from plants

Some of the laticiferous plants identified by Bhatia et al. (1983) were investigated in detail at Jaipur (for review see Kumar et al., 1995; Kumar 2000 and 2001) .

Certain potential plants were selected and attempts were made to develop proper agro-technology for their large scale cultivation. Initially work was initiated at 5 ha and subsequently extended to the 50 ha EPDPC.

Methodology employed

Certain potential plants were selected and attempts were made to develop agro-technology for their large scale cultivation (Kumar, 1984; 1994; Kumar et al, 1995; Kumar 1996; Kumar,1998; Roy, 1998 – for review see Kumar, 1995 and Kumar, 2000). A 50 ha bio-energy plantation demonstration project center has been established on the campus of the University of Rajasthan to conduct the experiments on large scale cultivation of selected plants with the objective of developing optimal conditions for their growth and productivity, besides conserving the biodiversity.

The work done included

i) Hydrocarbon yielding plants, ii) high molecular weight hydrocarbon yielding plants, (iii) non edible oil yielding plants

(I) Hydrocarbon yielding plants included :

1.*Euphorbia lathyris* Linn., 2. *Euphorbia tirucalli*. Linn., 3. *Euphorbia antisyphilitica*, Zucc.,4. *Euphorbia caducifolia* Haines.,5.*Euphorbia neriifolia* Linn, 6.*Pedilanthus tithymalides* Linn, 7.*Calotropis procera* (Ait.)R.Br.,8.*Calotropis gigantea(*Linn) R.Br.

II) High Molecular Weight Hydrocarbon Yielding Plants :

Parthenium argentatum Linn.

III) Non edible oil yielding plants

1. *Jatropha curcas*. 2. *Simmondsia chinenesis*

Considerable work has ben carried out on these plants (Kumar, 1987;1994; 1995; 1996; Kumar and Roy, 1996; Roy and Kumar, 1998; 1990). Investigations on several plant species have been carried out at our center including *Euphorbia lathyris* (Garg and Kumar, 1987a ; 1987b; 1989a; 1989b; 1990; Kumar and Garg, 1995) *Euphorbia tirucalli* (Kumar and Kumar, 1985, 1986;; Kumar and Kumar 1986) *Euphorbia antisyphilitica* (Johari et al., 1990b,1991; Johari 1992; 1993a; 1995) *Pedilanthus tithymaloides* (Rani et al. 1991;Rani and Kumar, 1994a); *Calotropis procera* (Rani et al, 1990) ; *Euphorbia neeriiifolia* and E. caducifolia (Kumar 1990, 1994) Jatropha curcas (Roy ,1990, 1991, 1992b, 1994, 1996; Roy and Kumar,1990) and *Simmondsia chinensis* (Roy, 1992a). The following aspects have been studied in detail:

(A) Propagation

In general these plants are easily propagated through cuttings. The optimum period for raising cuttings is June -July and March -April. Cuttings from the apical and middle portions of *E.antisyphilitica* exhibited 100 percent survival rate while none of the cuttings from the basal portions survived. Besides, cuttings treated with growth regulator IAA showed longest root length in a certain time period. Spacing among the planted cuttings is also a crucial factor for survival of cuttings. It was noted that initially up to a period of two months the survival percentage was maximum in closest planting density . However for better results in later stages they must be transferred to beds having a minimum distance of approximately 45 cm. At this optimum density productivity of *E. antisyphilitica* was the best. (Johari, 1992). Regarding environmental variations, the March to October period was best suitable for *E.antisyphilitica* because a linear increase in growth was recorded in the period (Kumar, 1990). During these months, maximum sprouting was observed in *Pedilanthus tithymaloides, E.antisyphiliitca* and *E.tirucalli*. Cuttings measuring around 15 cm in length and 1 cm in diameter gave optimal growth. Seeds of *Jatropha curcas* and *E.lathyris* also showed maximum germination during these months. Overall growth and productivity was lowest in the winter months from November to February. Higher accumulation of hexane extractables corresponded with higher temperatures of the summer season (Johari and Kumar, 1992).

(B) Edaphic factors

Among different soil types sand was best for the growth of *E. lathyris* (Garg and Kumar,1990) and *P. tithymaloides* (Rani et al., 1991) while red loam soil was best for *E. antisyphilitica.* However, for *E.lathyris* latex contents were maximum on sand gravel. Red soil was rich in nitrate, sodium, potassium and phosphorus pentaoxide (Johari and Kumar, 1992). *E. antisyphilitica* plants were relatively tall in sandy soil and less branched as compared to red soil. Plants grown in red soil branched more instead of increasing much in height. When different combinations of these soil types were made biomass of *E.antisyphilitica* was maximum in red loam+sand+gravel (Johari et al., 1990a). While the red loam+sand combination in equal amounts was best for *P.tithymaloides* (Rani et al., 1990). A mixture of gravel+sand favored maximum increase in plant height fresh weight and dry weight in *E. lathyris* (Garg and Kumar, 1990; Kumar and Garg, 1995). Environmental factors influenced the growth and yield of *Calotropis procera* (Rani et al.,1990)

(C) Growth Curve

Growth of these plants was promoted by relatively higher temperatures. Maximum growth was observed during June-July to October-November and also from February March to May June. Increase in hexane extractables (HE) was recorded up to 6-7 months; thereafter HE did not increase significantly in *E.lathyris. E.antisyphilitica* and *P.tithymaoildes*. Higher levels of HE were recorded in leaves as compared to the stem in *E. lathyris* and in fruits of *Calotropis procera*. The active phase of growth exhibited greater amounts of HE.

(D) Fertilizer application

Application of NPK singly or in various combinations improved growth of all the selected plants. In general NP combination gave better growth which was only slightly improved by the addition of K for *E.tirucalli*.(Kumar and Kumar, 1986). When best doses of NPK were applied in different combinations like NP, NK, KP and NPK the last combination gave best results in the form of biomass, latex yield, sugars and chlorophyll in *E.lathyris* (Garg and Kumar, 1990) and *P.tithymaloides* (Rani and Kumar, 1994a). In *E. antisyphilitica*, however, NP combination gave best results, followed by NPK for biomass production. Chlorophyll, sugars and latex yield was best in combination (Johari and Kumar, 1993a).

Addition of farm yard manure (FYM) alone and in combination with urea improved the growth and productivity of *E.antisyphilitica*, *E.lathyris* (Kumar and Garg, 1995), FYM + Urea application improved the productivity in comparison with FYM application alone. In *E.lathyris* addition of FYM increased the plant height fresh weight and dry weight to varying degrees. Hexane and methanol extractables also increased (Garg and Kumar, 1896; 1987a)

Micronutrients, B, Zn, Cu, Mn, Fe, and Mo were applied to *E.antisyphilitica*, *E. lathyris* and *P. tithymaoildes* in different concentrations. Their soil application resulted in general promotion in fresh and dry biomass, latex and chlorophyll yield. Foliar spray was given to E.lathyris. In this plant best results were obtained by Mg application followed by Cu, B, Fe, Mo, Zn and Mn (Garg and Kumar,1987a).

Salinity stress studies were also made on *Euphorbia tirucalli* (Kumar and Kumar, 1986). Salinity was applied in the form of irrigation water. Lower concentrations of salinity improved plant growth of *E. antisyphilitica* (Johari et al.,1990a) but higher concentrations inhibited further increase in growth. Sugars, however, did not increase in any saline irrigation. A slightly higher level of salinity impaired chlorophyll synthesis also. At higher level of salinity leaves of *E.antisyphilitica* became yellow and fell down but the stem did not show any visible adverse effects. *E. lathyris* could also tolerate lower salinity levels but its tolerance was lower than *E.antisyphilitica*. In *E.lathyris* salinity adversely affected root growth (Garg and Kumar, 1990). *P.tithymaloides* also exhibited increases in biomass and yield at lower salinity levels and higher concentrations adversely affected the plant. Its underground part could tolerate slightly higher salinity concentration (Rani et al., 1991). Saline irrigation was also given with different percentage of FYM added in the soil. Both *E.antisyphilitica* and *P.tithymaloides* exhibited tolerance of higher salinity levels with increasing percentage of FYM in the soil, biomass sugars, biocrude and chlorophyll all increased in proportion with increasing FYM levels in the soil and along with saline irrigation. It was found in *Euphorbia lathyris* that up to a certain level FYM causes increase in overall growth and yield along with different concentrations of saline irrigation. Beyond a certain level increased FYM did not improve growth and productivity. *P. tithymaloides* required still higher percentage of FYM in the soil for best yield and biomass.

Lower salinity levels increased the sugar contents in sand. Higher saline concentrations adversely affected the chlorophyll contents but with increase in manure supply the chlorophyll accumulation was promoted in *P.tithymaloides.* The effect of water stress was also studied. Five different percentages of field capacity (FC) were

determined and plants were irrigated. Above ground plant biomass improved significantly with increasing percentages of FC, maximum being 100 percent FC irrigation. In *E. antisyphilitica* as well as in *P. tithymaoildes* plant height also increased linearly with increasing soil water status. However, under ground length was found to increase up to a certain level only. Irrigation beyond an optimum level tended to reduce biocrude, sugar and chlorophyll in *E. antisyphilitica.* In *P. tithymaloides* lowest FC gave maximum yield of HE and chlorophyll. Sugar, however, increased with increasing levels of field capacity irrigation. Percent dry matter yield also decreased with increasing the quantity of irrigation water to the soil in *E.antisyphilitica* and *P. titymaloides* (Rani and Kumar, 1994a)

(E) Application of growth regulators

Exogenous application of growth regulators has been reported for several horticultural and ornamental plants and sugarcane. In *Euphorbia antisyphilitica* in the present experiment maximum plant height was observed with GA_3, followed by CCC, NAA, 2,4,5-T and IAA treatment. Spray of growth regulators resulted in enhanced fresh and dry weight production (Johari et al., 1994b). However bio-crude synthesis occurred more with the auxins NAA and IAA in *E. antisyphilitica*. Out of all the growth regulators employed on *P. tithymaoildes*, IAA supported maximum plant growth in terms of fresh weight and dry weight of above ground and under ground plant parts. 2,4,5-T showed minimum plant growth, and certain nodular structures were observed on the stem of the plants. Biocrude yield was best in IAA followed by 2,4,5-T, GA_3, CCC, NAA and control. Application of growth regulators on *P. tithymaloides* resulted in a slight decrease in chlorophyll, whereas on *E.lathyris* they induced favorable results regarding chlorophyll (Garg and Kumar, 1987a).

In *E.lathyris* IBA caused maximum fresh weight productivity followed by IAA, GA_3 and NAA. NAA sprayed plants exhibited more production of HE. A favorable influence of growth regulators was also observed in sugar yield maximum with NAA followed by IBA, GA_3 and IAA (Garg and Kumar 1987b).

The cultivation of these plants suffers from plant pathogenic diseases affecting at the root level. Investigations on pathogenicity and control aspects of Charcoal rot of *E.lathyris.* (Garg and Kumar, 1987c); *E.antisyphilitica* (Johari and Kumar, 1993b) were also carried out.

(F) Micropropagation

Plant tissue culture has been successfully employed to achieve rapid clonal propagation of *E.lathyris* (Kumar and Joshi,1982); *Pedilanthus tithymaloides*(Rani and Kumar,1994 b) and *E.antisyphilitica* (Johari and Kumar, 1994). Likewise propagation of jojoba has also been carried out (Roy 1972a). *Jatropha curcas* L. is potential diesel fuel yielding plant and details about this are given in Roy and Kumar, 1988 and Roy, 1999.

Development of wasteland

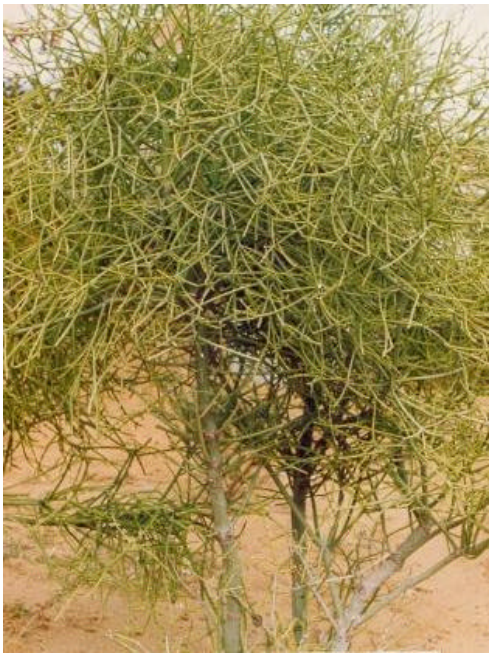
A protocol was set up for developing the wasteland following the three tier system in which small shrubs, shrubs and trees were used at a close spacing and this yielded a dry matter production of over 40 dry tonnes in a three year rotation .
The *Euphorbia antisyphilitica* in the lower tier, *Jatropha curcas* in the middle tier and *Acacia totilis* in the upper tier were used to colonize the EPDPC. The picture below represents the area as seen originally in Figure 1 and 2 which has been developed at EPDPC as greenland from the wasteland

(1)

(2)

(3)

(4)



(5)

(6)

(7)

(8)

(9)

(10)

(11)

(12)

Figure 1: Energy plantation Demonstration Projekt Center (EPDPC) University of Rajasthan Jaipur 1984. Barren Land with only one tree (Holoptelia integrifolia).

Figure 2: Another View of wasteland at EPDPC. Pitting was done at 1 Meter x 1 Meter For Plantation.

Figure 3: Euphorbia Antisyphilitica Nursery stage, with close spacings.

Figure 4: Calotropis Procera a hydrocarbon plant used to colonise

Figure 5: *Euphorbia tirucalli*

Figure 6: *Euphorbia caducifolia*

Figure 7: *Euphorbia neeriifolia*

Figure 8: *Jatropha curcas* and *Calotropis procera* in background

Figure 9: *Euphorbia antisyphilitica* and *Calotropis procera*

Figure 10: *Euphorbia antisyphilitica* and *Jatropha curcas* in background

Figure 11: Three tier system with *E.antisyphilitica* in foreground and *Acacia tortilis* in background

Figure 12: A well developed EPDPC following the three tier system.

Acknowledgement:

The constant encouragement of Professor Dr. K-H. Neumann is gratefully acknowledged. His frequent visits witnessed the development of the wasteland into greenland.

References

Alexandratos, N. (ed.) (1995): World Agriculture Towards 2010. FAO Rome.

Bhatia V. K., G.S. Srivastava, V.K.Garg, Y.K. Gupta, S. S. Rawat and J. Singh (1983): Study of laticiferous (latex bearing) plants as potential petro-crops. Fuel. 62, 953-955.

BP-AMOCO (ed) (1999): BP-AMOCO Statistical review of world energy 1998. BP-AMOCO Company, London.

Garg, J. and A. Kumar (1987b): Some studies on charcoal rot of Euphorbia lathyris caused by *Macrophomina phaseolina*. Indian Phytopathology 41, 257-260.

Garg, J. and A. Kumar (1987c): Effect of growth regulators on the growth, chlorophyll development and productivity of Euphorbia lathyris L.A hydrocarbon yielding plant. Progress in Photosynthesis Research. J. Biggins (ed.) IV (7), 403-406.

Garg, J. and A. Kumar (1989a): Potential petro crops for Rajasthan. J. Indian Bot. Soc., 68, 199-200.

Garg, J. and A. Kumar (1989b): Influence of salinity on growth and hydrocarbon yield of *Euphorbia lathyris*. J. Indian Bot. Soc. 68, 201-204.

Garg, J. and A. Kumar (1990): Improving the growth and hydrocarbon yield of Euphorbia lathyris L. in semi-arid regions of Rajasthan. In: Biomass for Energy and Industry Vol. I. (G. Grassi, G. Gosse and G. dos Santos, eds.) Elsevier Applied Science, London, pp. 1.527-1.531.

Garg, J. and A. Kumar, (1986): Studies on biomass production and improvement in biocrude content. Proc, Workshop on Petrocrops, New Delhi, pp. 69-81.

Garg, J. and A. Kumar, (1987a): Improving growth and hydrocarbon yield of Euphorbia lathyris L. In: Bioenergy Society Fourth convention and Symposium 87, R.N. Sharma, O.P. Vimal and A.N. Mathur, (eds.) New Delhi, pp. 93-97.

Hall, D.O. and Rosillo-Calle (1998): The role of bio-energy development. In: Biomass for energy and Industry. (H. Kopetz et al. (eds.), C.A.R.M.E.N. Germany, pp 52-55.

IEO (2001): International Energy Organisation Report – 20001.

Johari, S. and A. Kumar (1992): Effect of N. P. and K. on growth and biocrude yield of *Euphorbia antisyphilitica*. Annals of Arid Zone 31 (4), 313-314.

Johari, S. and A. Kumar (1993): Charcoal rot of Candelilla (*Euphorbia antisyphilitica* Zucc.) caused by *Macrophomina phaseolina* (Tassi) Goid. Indian J. Mycol. Plant Pathol. 23 (3), 317.

Johari, S. and A. Kumar (1994): *In vitro* propagation of *Euphorbia antisyphilitica*. J. Indian Bot. Soc. 60,

Johari, S. and A. Kumar, 1993. Charcoal rot of Candelilla (*Euphorbia antisyphilitica* Zucc.) caused by *Macrophomina phaseolina* (Tassi) Goid. Indian J. Mycol. Plant Pathol. 23 (3), 317.

Johari, S., S. Roy and A. Kumar (1990a): Influence of edaphic and nutritional factors on growth and hydrocarbon yield of *Euphorbia antisyphilitica* Zucc. In: Proc. Biomass for Energy and Industry. Vol. I. (G. Grassi, G. Gosse and G. das Santos, eds.). Elsevier Applied Science, London, pp. 1.522-1.526,

Johari, S., S. Roy and A. Kumar, 1991. Influence of growth regulators on biomass and hydrocarbon yield from *Euphorbia antisyphilitica* Zucc. In: Bioenergy for Humid and Semi-humid Regions (H.L. Sharma and R.N. Sharma, eds.) Bio-Energy Soc. of India, New Delhi, pp. 462-464.

Kumar, A. (2000b): Traditional Indian Ayurvedic Medicines. Some potential plants for bio-energy, medicine from India. Annual report : Institute of Natural Medicine. TMPU,Japan 27, 3-15.

Kumar, A. (l996): Bioenergy plantations: A model system for restoration of semi arid regions. In: Biomass for energy and environment (P. Chartier, G.L.Ferrero, U.M. Henius, S.Hultberg, J. Sachau, M.Wiinblad, eds.) Elsevier Science U K. pp 819-824.

Kumar, A. (1990): Prospects of raising latex bearing plants in semi-arid and arid regions of Rajasthan. In: Biomass for Energy and Industry, Vol. I. (G. Grassi, G. Gosse and G. dos Santos, eds.), pp. 1.100-1.107. Elsevier Applied Science, London.

Kumar, A. (1991): Growth and cultivation of latex bearing plant under semi-arid conditions of Rajasthan In: Proc. Bioenergy for Humid and Semi-humid Regions (H.L. Sharma and R.N. Sharma, eds.). Bioenergy Soc. of India, New Delhi, pp. 449-462.

Kumar, A. (1994): Laticifers as potential bioremedients for wasteland restoration. J. Environment & Pollution 1, 101-104.

Kumar, A. (1995): Cultivation of hydrocarbon yielding plants in Rajasthan as an alternative energy source. J. Env. and Pollution 2, 67-70.

Kumar, A. (1998): Biomass energy crops of semi arid regions of India and their energy potential. In: Biomass for Energy and Industry. (Kopetz H et al., eds.), C A R M E N. Germany pp. 345-348.

Kumar, A. (2000a): Hydrocarbon yielding plants and future prospects: Biotechnological approach. In: Plant Biotechnology- recent advances (P.C. Trivedi, ed.), Panima Publishing Company, New Delhi. pp 194-212.

Kumar, A. (2001): Conservation and utilization of Herbal drugs to protect them from extinction = an urgent need. Proceedings International Forum for Traditional Medicine. Toyama, Japan pp. 281-284.

Kumar, A. and J. Garg (1995): Effect of organic manures on growth and hydrocarbon yield of *Euphorbia lathyris* L. J. Environment & Pollution 2, 207-210.

Kumar, A., and S. Roy, (1996): Biomass resources of semi arid regions: Production and improvement of wood energy source. In: Biomass for energy and environment (P. Chartier, G.L.Ferrero, U.M. Henius, S.Hultberg, J. Sachau, M.Wiinblad, eds.) Elsevier Science, U.K. pp. 721-724.

Kumar, A., S. Johari, and S. Roy (1995): Production and improvement of bio-energy sources. J. Indian Bot. Soc. 74A, 233-244

Rani, A, S. Roy and A. Kumar, (1996): Effect of Salinity stress on growth and hydrocarbon yield of *Pedilanthus tithymaloides* var. Green. J. Environment & Pollution 3(1), 21-26.

Rani, A. and A. Kumar (1992): Comparative study on biomass production and hydrocarbon yield or three different varieties of *Pedilanthus tithymaloides*. Act. Ecol. 14, 77-79.

Rani, A. and A. Kumar (1994a): Effect of edaphic factors on the growth and physiology of *Pedilanthus tithymaloides* Var. Green. Journal of Environment and Pollution. 2(1), 5-8.

Rani, A. and A. Kumar (1994b): Micropropagation of *Pedilanthus tithymaloides* var. Green, a hydrocarbon yielding plant. J. of Phytol. Res. 7(1&2), 107-110.

Rani, A., S. Roy and A. Kumar (1990b): Influence of morphological and environmental factors on growth and hydrocarbon yield in *Calotropis procera*. In: Biomass for Energy and Industry. Vol. 1 (G. Grassi, G. Gosse, and G. das Santos, eds.) Elsevier Applied Science London, pp. 1.480-1.483.

Rani, A., S. Roy and A. Kumar (1991): Effect of salinity stress or growth and hydrocarbon yield of *Pedilanthus tithymaloides* variety Green (Linn.) Point. In: Proc. Bio-Energy for Humid and Semi-humid Regions (H.L. Sharma and R.N. Sharma, eds.), Bio-Energy Soc. of India, New Delhi, pp. 456-461.

Roy, S. (1996): Growth and productivity of non conventional energy sources: *J.curcas* In: Biomass for energy and environment (P. Chartier, G.L.Ferrero, U.M. Henius, S.Hultberg, J. Sachau, M.Wiinblad, eds.), Elsevier Science, U. K. pp. 560-565.

Roy, S. and A. Kumar (1990): Prospects of wood energy production in semi-arid and arid regions of Rajasthan. In: Proc. Biomass for Energy and Industry. (G. Grassi, G. Gosse and G. das Santos, eds.) Elsevier Applied Science, London, pp. 2.1153-2.1156.

Roy, S. and A. Kumar (1995): Biodiversity of Rajasthan and its energy potentials. J. Environment & Pollution. 2(3), 105-109.

Roy, S. and A. Kumar, (1998): Non edible oil and seed plants as source of energy and biodiesel. In: Biomass for energy and Industry. (Kopetz H. et. al., eds.), CARMEN. Germany, pp. 616-619.

STAFF, F (1998): Development of Biodiesel activity in France. In: Biomass for energy and industry. (H. Kopetz et al. ,eds.), CARMEN Germany, pp. 112-115

World Bank (1996): Rural energy and development : Improving energy supplies for 2 billion people. World Bank Industry and Energy Department Report No 1512 GLB. Washington.

Biochemical, Physiological and Morphological Responses of Sugar Beet to Salinization

Sayed S. Eisa and Safwat H. Ali

Departments of Agricultural Botany and Biochemistry

Faculty of Agriculture, Ain Shams University, Cairo, Egypt

Abstract

Biochemical, physiological and morphological responses of sugar beet grown on sandy soil under three levels of NaCl salinity in irrigation water, i.e. control, 3000 & 6000 ppm was studied in pot experiment. Results showed that root fresh weight linearly decreased by increasing NaCl salinity levels up to 6000 ppm, but sucrose percentage in root was significantly increased. On the other hand, increasing NaCl levels resulted in significant increase of Na content in both of shoot and root Meanwhile, K content in shoot was sharply decreased but K content in root didn't significantly differ by increasing NaCl levels. Also, under salinity results indicated a strongly negative correlation between shoot osmotic potential and shoot Na content while it was mainly with sucrose concentration in root. Thus, sugar beet plant has an active mechanism to include higher amount of Na in leaves and utilizes it to regulate leave osmotic potential under saline condition. Despite of this mechanism the transpiration rate and stomatal conductance showed significant decrease by increasing NaCl levels up to 6000 ppm. Moreover, stomatal behavior and stomatal morphology revealed a gradual response to the level of NaCl salinity used. Stomatal density, area and pore area strongly decreased by raising NaCl level from control to 3000 ppm with no effect on stomatal closure. No further response was shown for stomatal area by increasing NaCl from 3000 to 6000 ppm, while stomatal closure recorded 60% in lower & 30% in upper leaf surface at 6000 ppm NaCl level. Generally, it could be pointed out that the decrease of sugar beet root fresh weight at low salinity level (3000 ppm) may be due to osmotical stress while at high level of NaCl (6000 ppm) it was attributed to toxic effect of higher Na accumulation on photosynthesis which led to closed stomata in order to inhibit Na transport into leaves.

Introduction

Salinity is one of the principal abiotic factors affecting crop yield in arid and semi-arid areas**.** Because sugar beet has halophytic ancestors, *Beta vulgaris* ssp. *Maritima*, which is found in salt marshes so, sugar beet is a good target crop for studying in such salt-affected areas.
On the other hand, not enough knowledge of the biochemical and physiological basis of the detrimental effect of salt on growth and root yield of sugar beet is the main reason for the limited success of sugar beet planting under high salt condition. Therefore, intensive investigations have to be carried aimed at the understanding of the biochemical and physiological basis of sugar beet salt tolerance mechanisms in order to improve those mechanisms and consequently achieving salt tolerance not only of sugar beet but also of other conventional field crops.
Until now reduction of sugar beet root yield under salinization is unclear. However, many investigations suggested that this reduction is caused by inhibition of photosynthesis or nutrient deficiency or by mineral toxicity. Brugnoli and Bjorkman (1992) reported that the lowering of conductance to CO_2 diffusion caused by stomatal closure accounts for much of the reduction in photosynthesis under moderate salt stress. Also, Delfine *et al.* (1998) found that salt accumulation caused a drop of the Ca and Mg content in spinach leaves which might have decreased membrane stability and chlorophyll content respectively. Moreover, they concluded that salinity reduced photosynthesis primarily by reducing the diffusion of CO_2 to the chloroplast both by stomatal closure and changes in mesophyll structure, which decreased the conductance to CO_2 diffusion within the leaf. However, Very *et al.* (1998) reported that the halophyte *Aster tripolium* partially closes its stomata in response to high Na concentrations. Despite the fact that *Aster tripolium* possesses no specific morphological adaptation to salinity, this stomatal responses preventing excessive accumulation of Na within the shoot via the control of the transpiration rate, is probably a principal feature of observed salt tolerance within the shoot. On the other hand, Ali *et al.* (2000) summarized some of the salt tolerance mechanisms of sugar beet such as replacing most of the K in leaves by Na and selectivity of K over Na in young leaf as compared with the old leaf. Moreover, Flowers (1988) found that in genotypes with salt inclusion, the predominant strategy of osmotic adjustment is achieved by the accumulation of salts (mainly NaCl) in the leaf tissue, which is also indicated from the work of Warne *et al.* (1990) who reported that *Chenopodium rubrum* osmotical adjustment is achieved by accumulating electrolytes in leaves.

Materials and Methods

The present study was carried out at the Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Ten seeds of sugar beet (*Beta vulgaris* ssp. *vulgaris*) variety Top were sown on November 15th 1999 in pots (30 cm diameter with a bottom drainage hole) that were filled with 9.5 kg sandy soil (97.0 % sand, 1.6 % silt and 1.4 % clay).
Plants were thinned after 4 weeks to one plant per pot. Complete randomized design with ten replicates was used. Each replicate included three levels of NaCl salinity namely, control (tap water), 3000 and 6000 ppm. Salt treatments were added to irrigation water after 4 weeks from the sowing date and continued till the end of experiment. Modified nutrient solution after Arnon and Hoagland (1940) was used till 13 weeks from sowing. Plants were harvested 23 weeks after sowing, then immediately separated into shoot and storage root (root & crown at

the lowest leaf scar). Fresh weights of shoot and root were determined. Sucrose and glucose concentrations were determined in root and shoot fresh weight according to the method of Shaffer and Hartmann (1921). Sodium and potassium contents in sugar beet shoots and roots were measured using a flame photometer Petracourt PFP1 in the Biophysics Laboratory, Department of Biochemistry, Faculty of Agriculture, Ain Shams University. Osmotic potential of root and shoot material was estimated using the refractometric method described by Slavik (1974). Leaf temperature, transpiration rate and stomatal conductance were measured by a porometer L1-1600 (Licorginc, USA) on the leaf No.10 (from top to bottom of the plant). The morphological changes of stomata for leaf No.10 from different treatments were examined and calculated through a Joel Scanning Electron Microscope (T.33A) linked with the semafour software program in the Central Laboratory of the Faculty of Agriculture, Ain Shams University.

Statistical analysis

Statistical analysis was performed using the Statgraphics plus program, version 7 (1993). Means were compared using the least significant difference after Duncan (1955) at the 5% level of probability.

Results

Data in Table 1 show that the first level of NaCl (3000 ppm) resulted in a significant reduction of sugar beet root fresh weight to about 63 % of that found for the controls, while shoot fresh weight was not significantly affected. Raising the NaCl level to 6000 ppm lead to a significant reduction in both root and shoot fresh weight to 29.5 and 54.5 %, respectively, of that obtained in the control treatments. Consequently, root/shoot ratio was decreased linearly by increasing NaCl levels up to 6000 ppm in the nutrient medium.
Responses of sucrose and glucose content to increased NaCl levels detected for both root and shoot were varied (Table 1 & Fig.3). On the one side increasing NaCl levels up to 6000 ppm significantly increased root sucrose percentage but on the other, sucrose percentage in shoots showed no significant response. On the other hand, interesting results were shown for the glucose percentage in both shoot and root as affected by increasing NaCl levels in nutrient medium. Whereas glucose concentration increased in the roots it was reduced in the shoots by increasing NaCl level. This inverse responses of root and shoot glucose concentration to increased NaCl levels resulted in an obvious increases of root/shoot glucose concentration ratio.

Table 1: Effect of different levels of NaCl on sugar beet root and shoot fresh weight, root/shoot fresh weight ratio and sucrose concentration in roots (fw = fresh weight).

	Root	Shoot	Root/ shoot ratio	Root sucrose	Shoot sucrose	Sucrose root/shoot ratio
	(g plant^{-1} fw)			(g 100 g^{-1} fw).		
NaCl (ppm)						
Control	373a	110a	3.7	16.21c	0.55a	29.4
3000	235b	100a	2.7	18.03b	0.58a	31.1
6000	110c	60b	1.8	19.70a	0.62a	31.8

Means with the same letter in the same column are not significantly different at the 5% probability level.

.

The data in Table 2 show that increasing NaCl levels up to 6000 ppm significantly increased Na content for both root and shoot. However, increasing NaCl levels had no significant effect on root K content but shoot K content was significantly reduced. Another aspect of NaCl salinity effects on the distribution of K and Na between root and shoot is shown in Table 2. It's clear that higher amounts of K and Na in shoots than in roots were found even under salinization. However, the replacement of K by Na was more effective in leaves than in roots. Moreover, the selectivity of K in roots is more evident than in leaves, nevertheless K / Na ratio was decreased by increasing NaCl levels for both roots and shoots. In addition, the osmotic potential in either roots or shoots was significantly decreased as associated with increasing NaCl level in nutrient medium. However, in roots a lower osmotic potential (more negative value) was recorded than in shoot as shown in table 2.

Table 2: Effect of different levels of NaCl on sodium and potassium content, potassium / sodium ratio and osmotic potential of sugar beet roots and shoots (OP = Osmotic Potential , MPa = mega pascals)

Plant organ	Root				Shoot			
Parameters	K	Na	K/Na	OP	K	Na	K/N	OP
	(mg g^{-1} dry weight)			(MPa)	(mg g^{-1} dry weight)			(MPa)
NaCl levels (ppm)								
Control	8.0_a	0.2_c	40	- 1.2	52_a	2.2_c	24	- 0.32
3000	9.2_a	2.4_b	3.8	- 1.5	40_b	38_b	1.05	- 0.49
6000	10.0_a	3.4_a	2.9	- 1.7	30_c	65_a	0.46	- 0.58

Means with the same letter at the same column are not significant different at 5% probability level.

.

The data in table 3 show that the application of NaCl was followed by a reduction of both transpiration rate and stomatal conductance of leaf no.10. On the other hand, leaf temperature did not show any response to increasing salinity. However, the highest reduction of transpiration rate and stomatal conductance occurred at the higher level of NaCl. Whereas the values of transpiration rate and stomatal conductance at 3000 ppm NaCl were 34 and 37% less than in the control treatment, raising NaCl to 6000 ppm caused a reduction of these parameters by 47.5 and 50%.

Table 3: Effect of different levels of NaCl on temperature, transpiration rate and stomatal conductance of sugar beet leaf No.10 (from top to bottom).

	Leaf temperature (^oC)	Transpiration rate (mmol m^{-2} s^{-1})	Stomatal conductance (mmol m^{-2} s^{-1})
NaCl levels (ppm)			
Control	34_a	0.61_a	427_a
3000	34_a	0.40_b	270_b
6000	34_a	0.32_c	212_c

Means with the same letter at the same column are not significantly different at the 5% probability level

Concerning the morphological changes of stomatal criteria to salinity (Table 4), it is quite evident that each parameter observed exhibits its individual response to increased NaCl levels. Whereas stomatal density linearly decreased by increasing NaCl levels up to 6000 ppm, significant reduction of stomatal area was detected up to 3000 ppm and no further response was shown even at 6000 ppm. Stomatal closure showed another different response along with increased NaCl levels. This parameter showed no effective response concomitant with increasing NaCl up to 3000 ppm but stomata were closed at the highest NaCl level (6000 ppm). More focusing to the effect of salinity on stomatal closure for the upper (adaxial) and the lower (abaxial) leaf surface are shown in table 4. The distribution of stomatal density between the abaxial and adaxial leaf surface was decreased in a much more pronounced way for the abaxial than for the adaxial leaf surface as the NaCl level increased. Accordingly, the adaxial / abaxial distribution ratio of stomatal density was obviously decreased from 71.7 to 63.7 % by increasing NaCl level up to 6000 ppm. The opposite trend was true for stomatal pore area. Consequently, the closing of stomatal pores was more evident for abaxial than for the adaxial leaf surface. Results of stomatal closure (Table 4) supported the previous conclusion. The stomatal closure was firstly detected for the abaxial leaf surface at the lower level of NaCl (3000 ppm) while at 6000 ppm, stomatal closure was detected for both abaxial and adaxial leaf surfaces. However, stomatal closure was two times higher at the abaxial than at the adaxial leaf surface at the high NaCl level (6000 ppm).

Table 4: Effect of different levels of NaCl on stomatal density, stomatal area, stomatal pore area and stomatal closure for upper and lower surfaces of sugar beet leaf No.10 (from top to bottom). Stomatal closure (%) = No. of Stomata closed : No. of total stomata x 100. ND: Not detectable.

	Stomatal density (No. mm^{-2})			Stomatal area (μm^2)			Stomatal pore area (μm^2)			Stomatal closure (%)		
	Upper	Lower	Upper /lower	Upper	Lower	Upper /lower	Upper	Lower	Upper /lower	Upper	Lower	Upper /lower
NaCl (ppm)												
Control	81b	113a	71.7	530a	510a	104	40.0a	41.9a	0.95	ND	ND	ND
3000	60c	90.6b	66.8	364b	322b	113	29.8b	18.0c	1.7	1.0	11.0	0.1
6000	41.7d	65.5c	63.7	350b	360b	97	28.0b	11.0d	2.5	30	60	0.5

Means with the same letter for the same parameter are not significantly different at the 5% probability level.

Discussion

Starting with the responses of sugar beet yield to increased NaCl salinity levels up to 6000 ppm, it is clear that, despite of the linear decrease of root fresh weight nevertheless, root sucrose percentage was increased. At the same time, it was obvious that the reduction of root / shoot ratio along with increasing NaCl levels might be a modificative adaptation to reduce root mass. Consequently, root sucrose percentage was concentrated and thus root osmotic potential adjusted against high NaCl concentration around root system. However, the detrimental effects of salt on sugar beet yield have already been indicated by many investigators (Nassar, 1989; El-Hawary, 1990; Eisa, 1999). They reported that reduction of shoot as well as root fresh and dry weight were associated with increasing salinity levels. On the other hand, the ability of sugar beet to change its osmotic potential as a response to salt stress was discussed by Lindhauer *et al.* (1990) who reported that inorganic salts such as potassium, sodium and magnesium played the main role in osmotic potential adjustment in sugar beet leaves whereas sucrose dominated root osmotic potential. Also, results obtained in the present work revealed a more pronounced accumulation of both sucrose and glucose in the roots than in the shoots at increasing NaCl levels. Besides, a higher concentration of inorganic solutes (Na and K) were observed in shoots than in roots. However, correlation analysis (Figure 1) identified Na as the main solute for osmotic potential adjustment in sugar beet leaves under salinity conditions. Moreover, both sucrose and K are the main solutes for osmotic potential adjustment in roots followed by glucose and Na. Accordingly, it may be concluded that high Na concentration plays an important role in leaf metabolic function such as turgor maintenance. This ability to adjust shoot osmotic potential by using Na under saline condition is in agreement with characterizing sugar beet as a salt inclusion species.

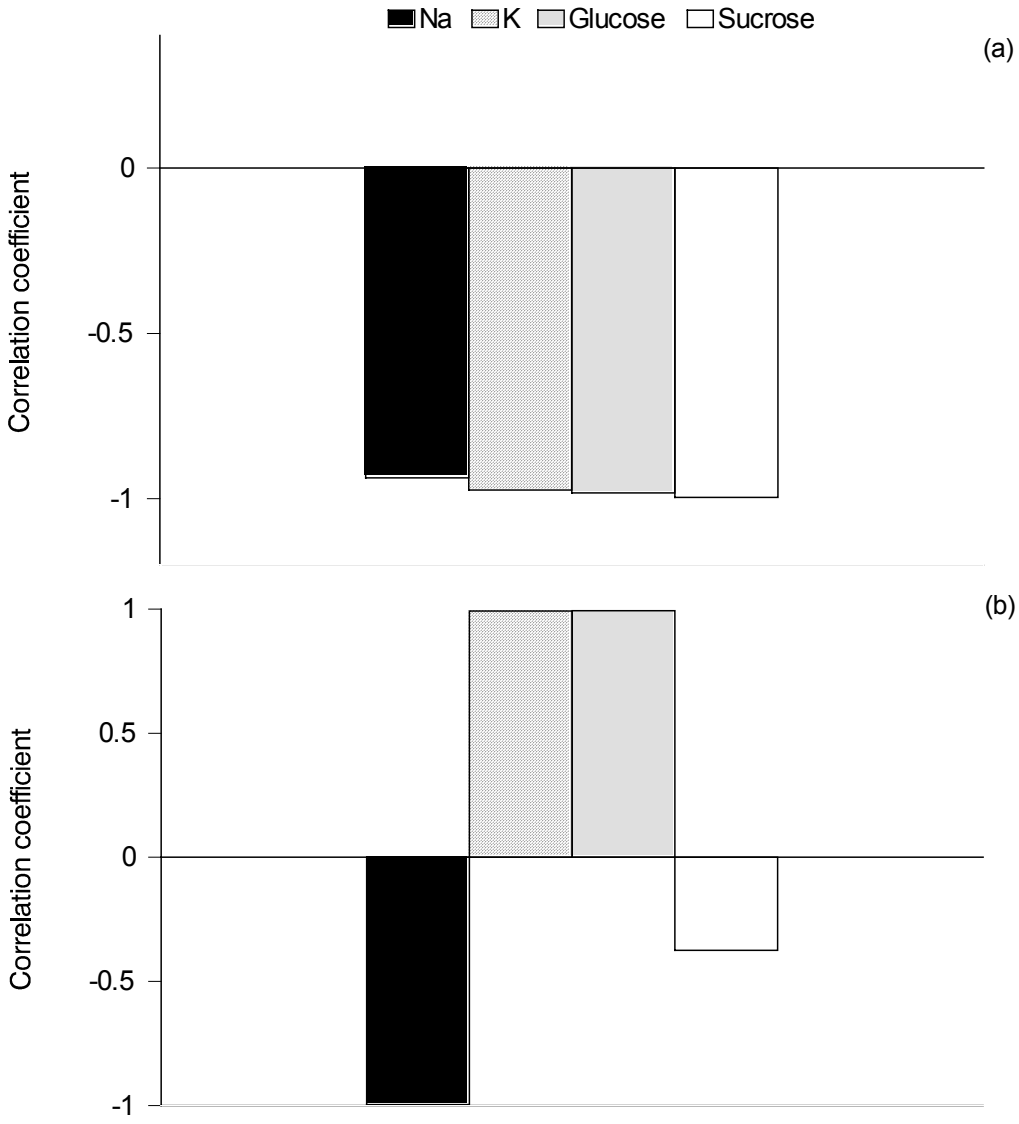


Fig.(1): Correlation coefficient between root (a) and shoot (b) osmotic potential and sodium, potassium, glucose and sucrose of sugar beet root and shoot under different concentrations of NaCl levels.

On the other hand, correlations presented in figure 2 illustrate that both K/Na ratio and osmotic potential in root and shoot were inversely correlated with stomatal closure whereas Na and sucrose content correlated proportionally with stomatal closure. It is clear that Na and sucrose content in both root and shoot led to a decrease of the osmotic potential which resulted in elevation of stomatal closure under saline condition. However stomatal closure correlated better with root osmotic potential (r = -0.9) than with that of the shoot (r = -0.8) and that may accentuate that stomatal closure was more a response to root osmotic potential than to that of the shoot under saline condition.

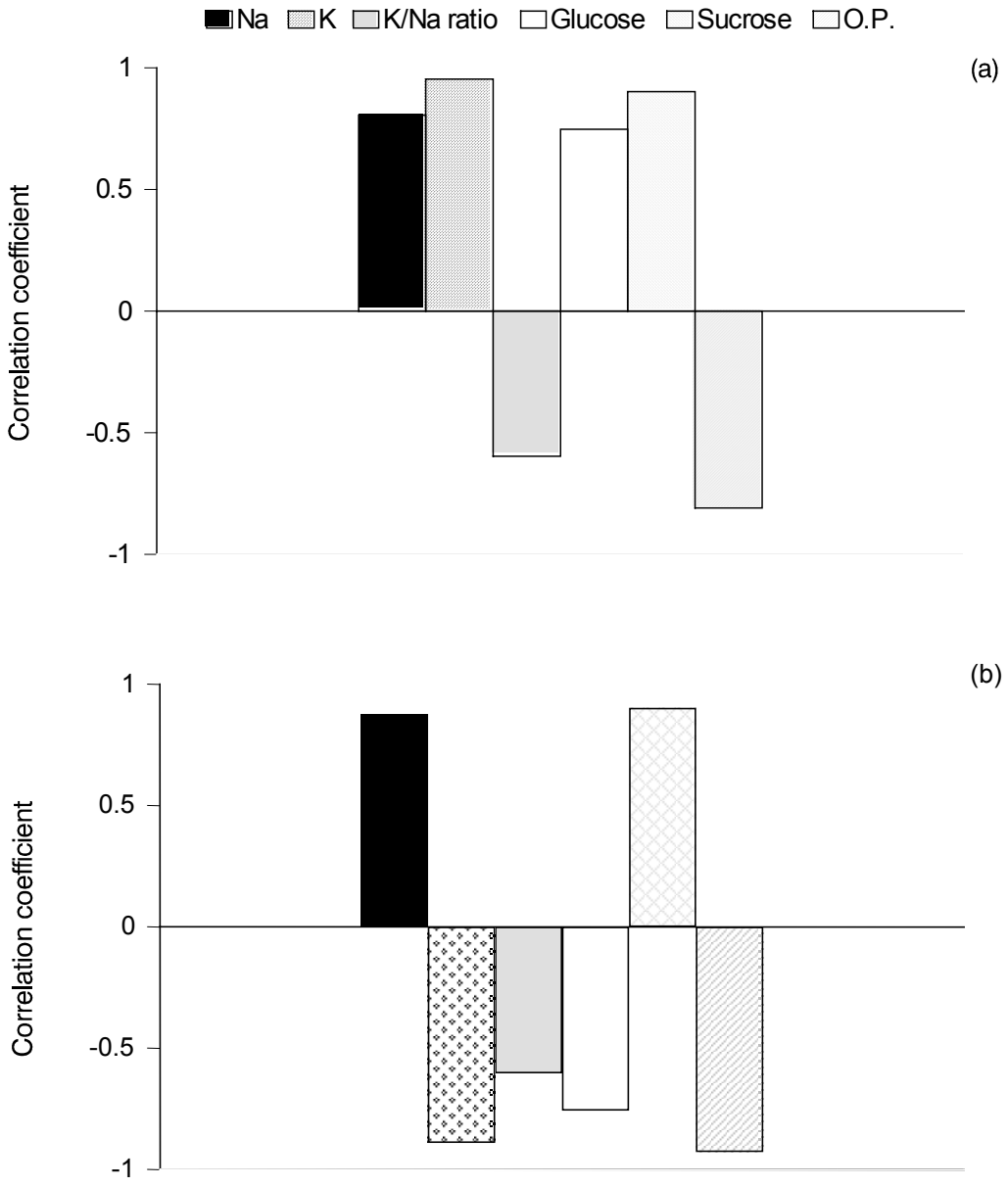


Fig.(2):Correlation coefficient between stomatal closure and sodium, potassium, K/Na ratio,glucose and sucrose of sugar beet root (a) and shoot (b) under different concentrations of NaCl levels.

Concerning the effect of increasing NaCl levels on sodium and potassium content, the present results show that increasing NaCl levels up to 6000 ppm greatly increased Na content in both shoots and roots, but sharply decreased K content in shoots (Table 2). On the other hand, root potassium content was not significantly affected by increasing NaCl levels. Accordingly, K / Na ratio, particularly for the shoot was obviously decreased. This pointed out a mechanism for replacing most of the potassium by sodium. As indicated by the higher amounts of Na accumulating in the shoots, an elimination of Na from the root by transportating it to the shoot where it replaces most of the K in leaves must be assumed. This mechanism (salt inclusion) has been detected by several investigators in some natrophylic plants to achieve salt tolerance (Greenway and Munns,1980; Gorham et al.,1985; Marschner, 1995); Haneklaus et al.,1998; Eisa, 1999; Ali et al. (2000).

As for biochemical aspects, it was evident that leaf glucose concentration sharply decreased by increasing NaCl up to 6000 ppm while the opposite response was observed for root glucose concentration (Figure 3). However, decreasing glucose concentration in the shoot and increasing concentration in the root as affected by salinity may favor sucrose transport and accumulation in the root. This might be reflected by a lower activity of acid invertase associated with high activity of sucrose–P synthase in leaves but the reverse was true in roots. Thus, it seems that under salinity stress sugar beet leaves are mainly a source while roots seem to be a main or the single sink as the formation of of new leaves was strongly reduced or even inhibited. On the other hand, control plants have two sinks, namely, young growing leaves and the root. This might be the second reason for increasing sucrose concentration in the root at increased salinity levels. Salt stress could be considered a factor helping plant leaves to shift from functioning as a sink to become a source by the aforementioned mechanism. Consequently, sucrose concentration increases in sugar beet roots. In this regard, Eschrich (1984) and Marschner (1995) reported that in sugar beet leaves the shift from sink to source is closely correlated with changes in enzyme activities associated with carbohydrate metabolism, namely a decrease in acid invertase activity (sucrose hydrolysis) and a sharp increase in sucrose–P synthase activity (sucrose synthesis). They added that sinks like young leaves and roots are characterized by high activities of acid invertase in the apoplasm. This enzyme hydrolyzes sucrose to form hexoses and thereby maintains a low sucrose concentration in the apoplasm. Consequently, sucrose transporting is enhanced by phloem unloading into the sink. Moreover, inhibitatited invertase activity in response to salinity was reported in sugar beet leaves (Rathert, 1982a), in cotton (Rathert, 1982b), and soybean (Rathert and Doring, 1983).

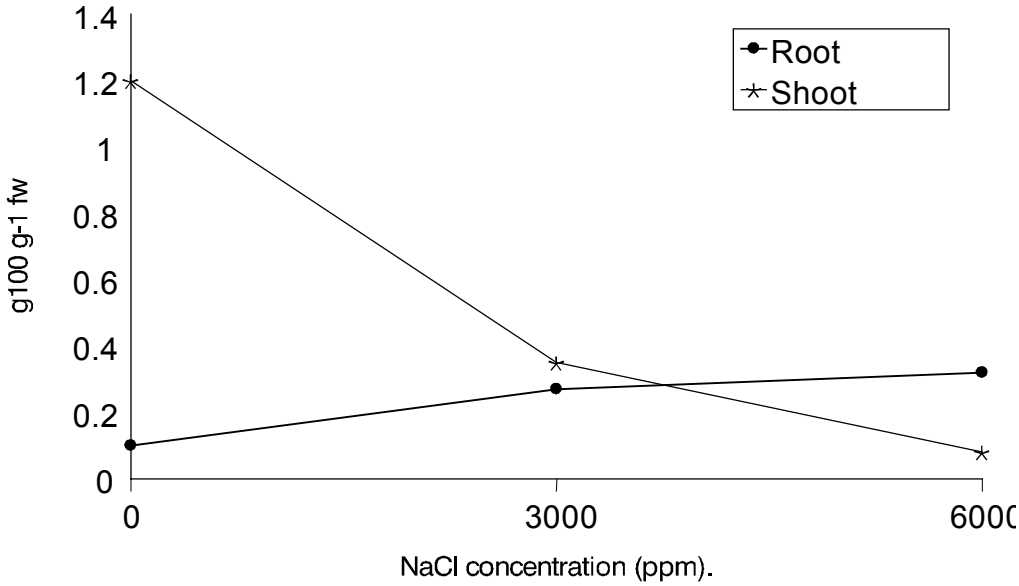


Fig. (3): Glucose concentration in root and shoot of sugar beet plant under two levels of (3000 and 6000 ppm NaCl)

Here again, concerning the effect of increasing NaCl levels on the K content in the root, the present data show slight accumulation of K content in the roots due to increased NaCl levels whereas K in the shoots decreased linearly at increasing NaCl levels. However, the accumulation of a suitable amount of K in root despite the increasing NaCl levels in the nutrient medium may have occurred due to a restricted transport of K from the root (sink) to the shoot (source).

This result was found to be in agreement with Wolf et al. (1991) who assumed that the high K and low Na concentration in young barley leaves and productive organs (sinks) were achieved by low xylem import of both K and Na but high phloem import of K from the mature leaves (source). However, the restriction of K transport into the shoot at increasing NaCl levels may be the second reason for K accumulation in the roots.

Accordingly, maintaining K at a suitable level in roots may be important for some essential metabolic functions in root such as enzyme activities. In this regard, Saftner and Wysc (1980) and Willenbrink *et al.* (1984) reported that in storage cells of sugar beet roots accumulation of sucrose is stimulated by potassium. Also, they added that sodium has an even greater stimulatory effect on sucrose accumulation.

Regarding the physiological and morphological responses to increasing NaCl levels, greatly reduced transpiration rate and stomatal conductance were observed by applying lower salinity level (3000 ppm NaCl) while a slighty further decrease for both parameters was recorded at the higher NaCl level (6000 ppm).

In the same regard, stomatal conductance was found to be sensitive even under mild salt stress either in salt sensitive beans (Gale et al., 1967) or resistant spinach (Robinson et al., 1983) or sugar beet plants (Heuer and Plaut, 1981). However, concerning the reduction of transpiration rate and stomatal conductance in the present work at the lower NaCl level (3000 ppm) it was attributed to a decrease in stomatal density, size and pore area but stomatal closure didn't play an important role at this level. On the other hand, higher NaCl level (6000 ppm) accounted for a sudden increase of stomatal closure. This sensitivity of stomatal closure at high NaCl (6000 ppm) may be the last mechanism for sugar beet plants to avoid the injuriuos effect of salt. In this regard, it could be suggested that photosynthesis seems to be strongly affected at high NaCl (6000 ppm) due to excessive accumulation of Na in leaves excerting toxic influences on this process. In other words, high transpiration rates lead to more accumulation of Na in leaves via xylem import which reaches the toxic level for active photosynthetic tissues and at this point stomata will be closed as the last way to avoid toxic effects of salt stress. Therefore, it may be concluded that the plant has been finishing off its aforementioned biochemical mechanisms such as replacement of K by Na in leaves.

Additional support that validates this suggestion can be obtained from an earlier report by Eisa (1999) who studied distribution and redistribution of Na in single cells of sugar beet leaves under various salinity levels, i. e. control (distilled water), 3000 and 6000 ppm NaCl. The author also reported that at the lower salt level (3000 ppm), Na accumulates in higher amounts in both upper and lower epidermis cells than in mesophyll cells. Thus, this partitioning mechanism within the leaf tissue eliminates the deleterious effect of Na from photosynthetically active tissue and this, in turn, reflects the insignificant effect of salt stress on the activity or concentration of CO_2 fixation enzymes. He added that, raising NaCl level up to 6000 ppm Na accumulates greatly in mesophyll cells because the epidermal cells were saturated which lead to a significant decrease in Rubisco concentration and increased PEPCase activity. From results presented it could be concluded that increasing PEPCase enzyme activity may an additional clue at limited CO_2 due to closed stomata at high NaCl (6000 ppm).

Conclusion

The present work has pointed out some salt tolerance mechanisms for sugar beet plant which could be summarized as following: Firstly, sugar beet plants have an effective inclusion mechanism through which sodium was readily translocated into the shoot where it replaced most potassium. Secondly, sugar beet plants have an ability to change the osmotic potential of shoot and root under saline condition. While sodium was mainly used for adjustment of shoot osmotic potential under saline condition, sucrose played a main role in the regulation of root osmotic potential followed by potassium, glucose and sodium against low osmotic potential in the nutrient medium. Therefore, increasing sucrose concentration in the root under saline condition may be attributed to the following: A) Decreasing glucose concentration in the shoot increased it in the root in order to orient sucrose translocation into the root. B) Preventing the induction of new leaves will keep the root to be the main sink. C) as a consquence of reduced root mass formation sucrose was concentrated in the root. Thirdly, the ability to regulate leaf transpiration rate and stomatal conductance by gradual control of stomatal behavior according to the salinity level innutrient medium: Primarily at lower NaCl (3000 ppm) the reduction of the transpiration rate was caused by reducing stomatal density, size and pore area but not attributed to stomatal closure while at a higher NaCl (6000 ppm), it was predominantly attributed to stomatal closure. Accordingly, it can be pointed out that the reduction of root fresh weight yield at low NaCl (3000 ppm) may be due to osmotical stress but at high level of NaCl (6000 ppm) to the toxic effect of the higher Na content on photosynthesis. Moreover, another ability to regulate leaf transpiration rate was detected as stomata started to close firstly at lower leaf surface at low level of NaCl (3000 ppm) and then in both leaf surfaces at the higher NaCl level (6000 ppm). However, at high salinity stomatal closure at the lower surface was two times more than at the upper leaf surface.

Acknowledgements

The authors wish to express their deepest gratitude to Professor Dr. S.A. Habib, Department of Agriculture Botany, Faculty of Agriculture, Ain Shams University, Cairo, Egypt, for his help, valuable suggestions and revision of this article.

References

Ali, S. H., I.Z. El-Shamey and S.S.Eisa (2000): Sodium potassium balance and adaptation of sugar beet to salt stress. Conf. Agric. Dev. Res. Fac. Agric. Ain Shams Univ, Cairo, Egypt, Annals Agric. Sci., Special Issue 2000, 1, 41-56.

Arnon, D. I. and D.R. Hoagland (1940): Crop production in artificial solutions and soil with special references to factor influencing yields and absorption of inorganic nutrients. Soil Sci. 50, 430-435.

Brugnoli, E. and O. Björkman, (1992): Growth of cotton under continuous salinity stress: influence on allocation pattern, stomatal and non-stomatal component of photosynthesis and dissipation of excess light energy. Planta. 187, 335-347.

Delfine, S., A. Alvino, M. Zacchini and F. Loreto (1998): Consequences of salt stress on conductance to CO_2 diffusion Rubisco characteristics and anatomy of spinach leaves. J. Plant Physiol. 25, 395-902.

Duncan, D. B. (1955): Multiple range test and multiple F test. Biometries. 11, 1-42.

Eisa, S. S. (1999): Optimization of sugarbeet nutrition in sandy soil. Ph.D. Thesis, Fac. Agric., Ain Shams Univ. Cairo, Egypt. pp 50-60.

El-Hawary, M. A. (1990): Effect of soil salinity and nitrogen fertilization on sugar beet. Ph.D. Thesis. Fac. of Agric.Al-Azhar Univ., Cairo. Egypt.

Eschrich, W. (1984): Untersuchungen zur regulation des Assimilattransport. Ber. Dtsch. Bot. Ges. 97, 5-14.

Flowers, T. J. (1988): Chloride as a nutrient and as an osmoticum. In: Advances in Plant Nutrition; Praeger, New York. 3, 55-78.

Gale, J., H.C. Kohl and R.M. Hagan (1967): Changes in the water balance and photosynthesis of onion, bean and cotton plants under saline conditions. Physiologia Plantarum 20, 408-420.

Gorham, J., R.G. Wyn Jones and E. McDonnell (1985): Some mechanisms of salt tolerance in crop plants. Plant and Soil. 89, 15-40.

Greenway, H. and R. Munns (1980): Mechanism of salt tolerance in monohalophytes. Annu. Rev. Plant Physiol. 31, 149-190**.**

Haneklaus, S., L. Knudsen and E. Schnug (1998): Relationship between potassium and sodium in sugar beet. Communications in Soil Science and Plant Analysis. 29 (11-14), 1793-1798.

Heuer, B. and Z. Plaut (1981): Carbon dioxide fixation and ribulose-1,5-bisphosphate carboxylase activity in intact leaves of sugar beet plants exposed to salinity and water stress. Annals of Botany 48, 261-268.

Lindhauer, M.G. Haeder, H. E. and Beringer, H. (1990): Osmotic potentials and solute concentrations in sugar beet plants cultivated with varying potassium/sodium ratios. Z. Pflanzenernähr. Bodenk. 153, 25-32.

Marschner, H. (1995): Mineral nutrition of higher plants. 2nd Ed. Academic Press, pp. 660-680.

Nassar, Z. M. (1989): Salt tolerance in fodder beet. Ph.D. Thesis, Fac. of Agric., Cairo Univ., Egypt.

Rathert, G. (1982a): Influence of extreme K:Na ratios and high substrate salinity on plant metabolism of crops differing in salt tolerance. V. Ion. Specific salinity effects on invertase in leaves of bushbean and sugarbeet plants., J.Plant Nut. 5, 97-109.

Rathert, G. (1982b): VII. Relations between carbohydrates and degradative enzymes in salt tolerant and salt sensitive cotton genotypes during initial salt stress. J. Plant Nut. 5, 1401-1413.

Rathert, G. and H.W. Doring (1983): VIII. Ion-specific salinity effects on carbohydrates and degradative enzymes of different salt-tolerant soybean varieties. J. Plant Nutr. 6, 583-595.

Robinson, S.P., W.J.S. Downton,. and J.A. Milhouse (1983): Photosynthesis and ion content of leaves and isolated chloroplasts of salt-stressed spinach. Plant Physiol. 77, 238-242.

Saftner, R. A. and R.E. Wyse (1980): Alkali cation / sucrose co-transport in the root sink of sugar beet. Plant Physiol. 66, 884-889.

Shaffer, P.A. and A.F. Hartmann (1921): The iodometric determination of cupper and its use in sugar analysis (modified by E. Noakell and A. El-Gawadi; Barnell, New Phytol. 35: 229-266). J. Bio. Chem. 45, 365-370.

Slavik, B. (1974): Methods of studying plant water relations. 9, 104-109. Academia Publishing House of the Czechoslovak Academy of Sciences Prague.

Very, A.A., M.F. Robinson, T.A. Mansfield and D. Sanders (1998): Guard cell cation channels are involved in Na induced stomatal closure in a halophyte. Plant J. 14 (5), 509-521.

Warne, P., R.D. Guy, L. Rollins and D.M. Reid (1990): The effect of sodium sulphate and sodium chloride on growth, morphology, photosynthesis, and water use efficiency of *Chenopodium rubrum*. Can. J. Bot. 68 (5), 999-1006.

Willenbrink, J., S. Doll, H.P. Getz and S. Meyer (1984): Zuckeraufnahme in isolierten Vakuolen and Protoplasten aus dem Speichergewebe von Beta-Rüben. Ber. Dtsch. Bot. Ges. 97, 24-39.

Wolf, D., R. Munns, M.L. Tonnet and W.D. Jeschka (1991): The role of the stem in the partitioning of Na and K in the salt treated barley. J. Exp. Bot. 42: 697-704.

Availability of Heavy Metals in Soils and their Uptake by Vegetable Species[+]

Sigrid Dieckmann, Olaf Melzer and Ludwig Bender[*)]

Fachhochschule Osnabrück - University of Applied Sciences, Department of Agricultural Sciences, Oldenburger Landstraße 24, D-49090 Osnabrück

[*)] Corresponding author; Fax: + (49) 541 5170; e-mail: l.bender@fh-osnabrueck.de

Abstract

Head lettuce, bush beans and celery were grown in subsequent years in an experimental field on anthropogenously uncontaminated or heavy metal contaminated soils at a mean pH of 6.3 ± 0.1. The contaminated plots were made up by amending or replacing the upper 20 cm soil layer with heavy metal contaminated alluvial top soil. Contamination includes Cd, Zn, Pb and Cu. Phytoavailable fractions of these elements were extracted with 1 M ammonium nitrate. Cd, Zn, Pb and Cu were also determined in the leaves of all three species as well as in bean pods and celery bulbs. The results show that plant uptake of Cd and Zn increased with increasing soil contamination while the uptake of Pb was low. No dependence of Cu uptake on total soil Cu content could be seen within the given contamination range. The relationship between Cd, Zn, Pb and Cu in plants and ammonium nitrate soil extracts was determined by twofactorial linear regression where r was approximately 0.8 for Zn, 0.7 for Cd and 0.5 for Pb whereas no relationship was found for Cu.
For the pot experiments carried out in a greenhouse two different soils (loamy sand, silt loam) were amended with 5 and 10 % metallurgical slag and adjusted to pH levels around 7 and 5 while controls did not contain slag. Phytoavailable heavy metal fractions were extracted from soil samples using ammonium nitrate or Calcium chloride + DTPA (CAT). Spinach was grown on these soils and the concentrations of Cd, Zn, Pb and Cu in the shoots correlated with their concentration in the soil extracts. The uptake of these elements by the plants increased with increasing slag amendment and decreasing pH. Strong depression of growth was observed at a pH around 5 in all treatments of the lighter and in the slag treatments of the heavier soil. In the slag treatments this was accompanied by increased endogenous Cd and Zn concentrations. The plant content of Cd, Zn and Pb correlated better with the ammonium nitrate extractable soil fraction of these elements than in the field experiments ($r > 0.9$). Correlations based on the CAT extractable fraction of Cd and Zn yielded values for r around 0.5 while it was only 0.1 for Pb. As in the field trials correlations for Cu were very poor with both extraction methods.

[+] This paper contains in part data from the undergraduate thesis work of the following students: Lars Schade, Andrea Kampf, Ralf Krämer, Ursula Veenker, Kirsten Homola, Axel Milius, Kathrin Eichholz and Dagmar Lehmann.

Introduction

The uptake of toxic heavy metals from contaminated soils by food and forage plants comprises a prominent path for such elements to enter the food chain and will finally be ingested by humans. Ingestion and eventual accumulation of toxic heavy metals pose a threat to human health and should, therefore, be minimized (Kloke, 1988; Traulsen and Schönhard, 1995; Delschen and Rück, 1997). It was also reported that the uptake of heavy metals by crop plants may vary in different cultivars of a particular species (Lübben, 1991; Metz and Kloke, 1998).
The total content of a given heavy metal in soils is considered impractical for the prognostication of its uptake by plants because only certain fractions of an element are phytoavailable (Brümmer et al., 1986; Brümmer and Hornburg, 1989; Birke and Werner, 1991). Thus, it has been suggested that soil heavy metal fractions available to plants are determined using salt solutions, e.g. 0.1 M calcium chloride (Köster and Merkel, 1982), 1 M ammonium acetate (Dües, 1989) or 1 M ammonium nitrate (Prüeß, 1992). The ammonium nitrate extractable fraction is considered mobil and readily available to plants whereas the ammonium acetate extract is assumed to include a fraction which can easily replenish the available fraction (Zeien, 1995). The so called CAT method, which employes a mix of 0.01 M calcium chloride and 0.002 M DTPA (Diethylen-triamine-pentaacetate) was suggested for the determination of macro- and micronutrients in horticultural substrates (Alt and Peters, 1993; Alt et al., 1994). Also, availability is influenced by a variety of parameters, of which soil pH is certainly the most important. Depending on the particular metal, decreasing pH increases the unspecifically adsorbed (available) fraction to various degrees while the specifically adsorbed (unavailable) fraction decreases accordingly. While e.g. Cd and Zn become increasingly available starting at pH 6.5 or 6, respectively, and are thus classified as rather mobil and more readily available elements, the corresponding pH for Cu is 5 - 4.5 and for Pb 4 –3.5 which classifies them as rather immobil and unavailable at least within the pH range of most arable and grassland soils (Hornburg et al., 1995). Slags from metallurgical or waste incineration plants etc. are frequently used in landscape construction or hydraulic engineering. They reportedly contain heavy metals in chemically very inert binding forms (Lahl, 1994; Khorasani, 1999). However, if exposed to environmental influences or if mixed with soils, chemical processes such as pH changes may alter the chemical binding of metals and eventually increase their mobility and bioavailability.

In the field experiments reported here we studied the uptake of Cd, Zn, Pb and Cu by a number of vegetable species in field experiments on soils of comparable pH but different degrees of heavy metal contamination. In these experiments four cultivars of each vegetable species were tested also for differences in the uptake of the heavy metals mentioned above. In pot experiments carried out in a greenhouse spinach was grown at two pH levels on two different soils amended with varied amounts of metallurgical slag or whithout slag. The correlation between soil heavy metal concentrations extractable with ammonium nitrate or CAT and the uptake by the vegetable species tested was assessed by twofactorial linear regression. Consequences on growth and heavy metal accumulation as well as the usfulness of these extractants for the prognosis of heavy metal transfer to plants are discussed.

Material and methods

Soils

The experimental field comprises 48 plots 1 x 2 m in size which are rimmed by concrete liners. The soil is classified as loamy sand (4 % clay, 30 % silt, 66 % sand) with 2 % organic matter and a pH of 6.2. 16 plots were left unchanged and designated *Soil 1*. Another 16 plots were made up by removing the top soil to a depth of 20 cm and replacing it by a 3 : 1 mix of original top soil as described above and alluvial top soil contaminated with Cd, Zn, Pb and Cu. The mixing resulted in a sandy loam (9 % clay, 35 % silt, 56 % sand) with 3.5 % organic matter and a pH of 6.4 which was designated *Soil 2*. In the remaining16 plots the top soil was replaced by an unmixed charge of the same alluvium (silt loam, 20 % clay, 58 % silt, 22 % sand) with 10 % organic matter and a pH of 6.3, designated *Soil 3*. Plots were arranged in randomized order. Using the described technique of soil replacement it could be avoided to amend the plots with heavy metal salt solutions which would have caused unrealistically high availability of metal ions until a natural equilibrium between specifically and unspecifically adsorbed fractions was attained.

For the pot experiments carried out in a greenhouse loamy sand as described above (Soil A) and silt loam consisting of 17 % clay, 74 % silt, 9 % sand (Soil B) were used. Portions of these soils were amended with 5 or 10 % ground metallurgical slag (particle size 0.5 to 1 mm) while a third portion of each soil remained free of slag. The soils were then adjusted to pH levels around 7 or 5, respectively, by either liming with Ca carbonate or a mixing-in of diluted sulfuric acid (0.02 to 0.5 M, depending on requirement). These preparations resulted in six different treatments for each soil designated A5/0, A5/5, A5/10 and A7/0, A7/5, A7/10 as well as B5/0, B5/5, B5/10 and B7/0, B7/5, B7/10. A or B indicate the soil, the first number shows the pH level and the second the slag amendment in % (w/w). Five pots per treatment with 12 kg soil each were set up amounting to a total of 60 pots. The actual pH ranged from 6.7 to 8.0 at the higher and from 4.9 to 5.6 at the lower end. Due to the presence of the basic slag the pH had to be checked and readjusted immediately before seeding and was also measured for variations right after harvest. It should be noted here that pH adjustment was very difficult in the beginning, however, it had stabilized acceptably after about 4 years.

Plant material

Head lettuce (Lactuca sativa, var. Capitata, Chichoriaceae, cultivar Mirian, Martina, LM 8015 and Floret), bush beans (Phaseolus vulgaris, var. nanus, Fabaceae, cultivar Modus, Pfälzer Juni, Nickel and Xavo) and celery (Apium graveolens, var. Rapaceum, Umbelliferae, cultivar Ofir, Prinz, President and Monarch) were used in the field experiments in three subsequent years. Head lettuce was harvested and analyzed in a marketable stage. Bean leaf and pod samples were taken at three developmental stages (1, anthesis; 2, first harvest of green pods three weeks after anthesis; 3, final harvest of green pods five weeks after anthesis). Celery was harvested upon development of marketable bulbs. Peeled bulb segments as well as peel and leaf samples were analyzed separately. In the pot experiments metal uptake by spinach (Spinacea oleracea, var. inermis, cultivar Laska) leaves was studied and correlated with the content of these metals in soil extracts obtained with two different salt solutions. All plant organs were thoroughly washed including a final rinse with distilled water before they were further processed.

Analytical

For the determination of the total heavy metal content of the soils 0.5 - 1 g air dried and ground soil was digested in quartz vials with 6 ml nitric acid (65 %), 2 ml hydrochloric acid (37 %) and 0.25 ml hydrogen peroxide (35 %) in a microwave digestion apparatus (MLS – Ethos plus; Mikrowellen-Laborsysteme, D-88299 Leutkirch).
Heavy metal fractions were extracted from 20 g air dried soil using 50 ml 1 M ammonium nitrate or 50 ml 0.01 M Calcium chloride + 0.002 M DTPA (pH 2.6 – 2.65; further referred to as CAT) for 1 h on a gyratory shaker.
The microwave technique and quartz vials were also used for the digestion of plant material (0.2 to 0.5 g dry matter) with 2.5 - 5 ml nitric acid (65%).
Certified soil (GBW 07404, Breitländer GmbH, D-59077 Hamm) and plant standard reference material (spinach leaf no.1570a, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA) were used to check the efficiency and precision of digestion procedures and measurements. However, no soil standard reference material certified for the extraction of element fractions with salt solutions was available. Therefore, uncertified soil samples from our own stocks with well determined contents of the elements in question were used as reference material with these analyses. Heavy metal concentration was principally measured emploing inductively-coupled plasma – optical emission spectrometry (ICP-OES, Perkin Elmer Optima 3300 XL with axial plasma viewing) while for the determinaton of lowest element concentrations graphite tube atomic absorption spectrometry (GT-AAS, Perkin Elmer SIMAA 6000) was used with a palladium-magnesium nitrate modifier to attain optimum analyte stability and atomization conditions.

Results

Field experiments

Total and ammonium nitrate extractable Cd, Zn, Pb and Cu of the three soils are given in table 1. Data derived from soil 1 are typical for anthropogeneously uncontaminated soils. The contamination of soils 2 and 3 is moderate except for the high Zn content of soil 3. It should be noted, however, that the limits set by german authorities for the application of sweage sludge (Cd 1.5, Zn 200, Pb 100 and Cu 60 mg kg^{-1}soil; AbfallKlärV, 1992) were exceeded for Zn and Pb in soil 2 and for all four elements in soil 3. Based on total contents, ammonium nitrate extractable fractions amounted to about 10 - 12 % for Cd, 5 - 6 % for Zn and 1 - 2 % for Pb and Cu at the given soil pH. Despite the increase of total Cu content ammonium nitrate extractable Cu was nearly the same in all three soils.
The content of Cd, Zn, Pb and Cu in head lettuce, beans and celery are given in table 2. Since in the bean experiment no significant differences were found at the three developmental stages investigated, only data from developmental stage 2 (first harvest of green pods) are presented. Cd and Zn increased in all three species with increasing soil contamination. The Cd content of the edible parts of lettuce and celery were about the same on soil 1, however, celery bulbs accumulated significantly more Cd than lettuce on the anthropogenously contaminated soils 2 and 3. Celery leaves contained slightly less Cd than the bulbs. While Cd uptake by beans was about one order of magnitude lower than in the other two species, the content in leaves was two to three times higher than in pods where it amounted to only about 0.1 mg kg^{-1} DW even on soil 3. Highest Zn levels were found in lettuce and celery leaves which amounted to more than 300 and 400 mg kg^{-1} DW, respectively, on soil 3. Celery bulbs contained between 20 and 30 % less Zn than leaves. Despite the high Zn accumulation on the two contaminated soils no toxicity symptoms were observed in either species. Beans, on the other

hand, exhibited Zn contents that were about an order of magnitude lower than in lettuce and celery, however, no differences could be observed between leaves and pods. The uptake of Pb by all three species was low on all soil contamination levels, as expected at the given pH. A slight increase in lettuce as well as in celery leaves and peels was observed with increasing soil contamination but no Pb could be detected in the peeled celery bulbs. It should also be noted that bean pods contained about 25 % less Pb than bean leaves. The similar amount of ammonium nitrate extractable Cu in all three soils is reflected by the Cu uptake of the plants which appeared to be independent of the total soil content. However, Cu content in plant material varied with species and also organs. Lettuce contained about 8 to10 mg kg^{-1} DW. Bean leaves exhibited approximately 17 and pods 11 mg. While celery leaves contained only 5 to 8 mg it amounted to13 mg in bulbs. Regarding the uptake of the elements under investigation no significant differences between the cultivars of either species were found.

Cd and Zn fractions extracted from soils using 1 M ammonium nitrate correlated fairly well with the plant content of these elements. The correlation for Pb, on the other hand, is rather poor and no relationship could be demonstrated for Cu. Since these findings were similar for all three vegetable species, only the results for lettuce are given in figure 1 as an example.

Table 1: Total and ammonium nitrate extractable heavy metal content (mg kg^{-1} DW) of the three soils used in the experimental field studies. Data are mean ± SD (n = 16).

	Cd	Zn	Pb	Cu
		Total		
Soil 1	0.36 ± 0.03	66.6 ± 6.4	22.9 ± 1.5	15.0 ± 0.6
Soil 2	1.23 ± 0.05	359.8 ± 18.0	111.1 ± 5.7	34.8 ± 1.8
Soil 3	3.91 ± 0.41	1204.6 ± 112.3	414.2 ± 42.7	77.6 ± 7.7
		Ammonium nitrate extractable		
Soil 1	0.05 ± 0.01	4.16 ± 1.17	0.30 ± 0.12	0.30 ± 0.12
Soil 2	0.15 ± 0.04	22.14 ± 7.23	2.01 ± 0.52	0.44 ± 0.11
Soil 3	0.34 ± 0.06	59.05 ± 12.18	3.21 ± 0.79	0.55 ± 0.11

Table 2: Heavy metal content (mg kg^{-1} DW; b.q., below quantification limit) of head lettuce, bush beans and celery grown on soils with increasing heavy metal levels (Soil 1 anthropogeneously uncontaminated).
Data are mean ± SD (n = 16).

	Cd	**Zn**	**Pb**	**Cu**
Head lettuce				
Soil 1	0.77 ± 0.09	144.2 ± 26.2	2.73 ± 0,58	7.4 ± 0.7
Soil 2	0.98 ± 0.18	181.0 ± 14.1	3.11 ± 0.38	7.9 ± 0.4
Soil 3	1.56 ± 0.26	329.8 ± 33.4	5.16 ± 0.54	9.9 ± 1.4
Bean leaves				
Soil 1	0.05 ± 0.02	29.8 ± 5.6	2.61 ± 0.40	16.8 ± 2.3
Soil 2	0.09 ± 0.03	48.0 ± 11.2	2.73 ± 0.40	16.8 ± 1.4
Soil 3	0.25 ± 0.05	67.0 ± 18.8	2.24 ± 0.29	16.1 ± 2.8
Bean pods				
Soil 1	0.02 ± 0.008	44.3 ± 7.2	1.81 ± 0.05	10.2 ± 1.3
Soil 2	0.04 ± 0.02	54.4 ± 6.5	1.83 ± 0.24	11.0 ± 1.0
Soil 3	0.08 ± 0.03	62.9 ± 11.4	1.88 ± 0.17	11.2 ± 1.9
Celery leaves				
Soil 1	0.41 ± 0.20	117.4 ± 38.8	0.92 ± 0.28	4.9 ± 0.7
Soil 2	0.92 ± 0.24	199.1 ± 42.2	1.61 ± 0.56	5.4 ± 0.7
Soil 3	2,08 ± 0,43	434,8 ± 56,0	1,70 ± 0,34	7,6 ± 1,4
Celery bulbs (peeled)				
Soil 1	0.63 ± 0.20	90.7 ± 15.8	b.q.	12.8 ± 2.9
Soil 2	1.32 ± 0.34	146.2 ± 18.7	b.q.	14.9 ± 2.7
Soil 3	2.90 ± 0.70	256.4 ± 37.0	b.q.	15.5 ± 2.6
Celery peels				
Soil 1	0.55 ± 0.13	95.9 ± 20.2	0.30 ± 0.15	9.4 ± 1.5
Soil 2	1.26 ± 0.23	174.5 ± 27.7	1.36 ± 0.52	11.8 ± 1.6
Soil 3	2.78 ± 0.43	318.9 ± 42.3	3.07 ± 1.30	14.3 ± 1.9

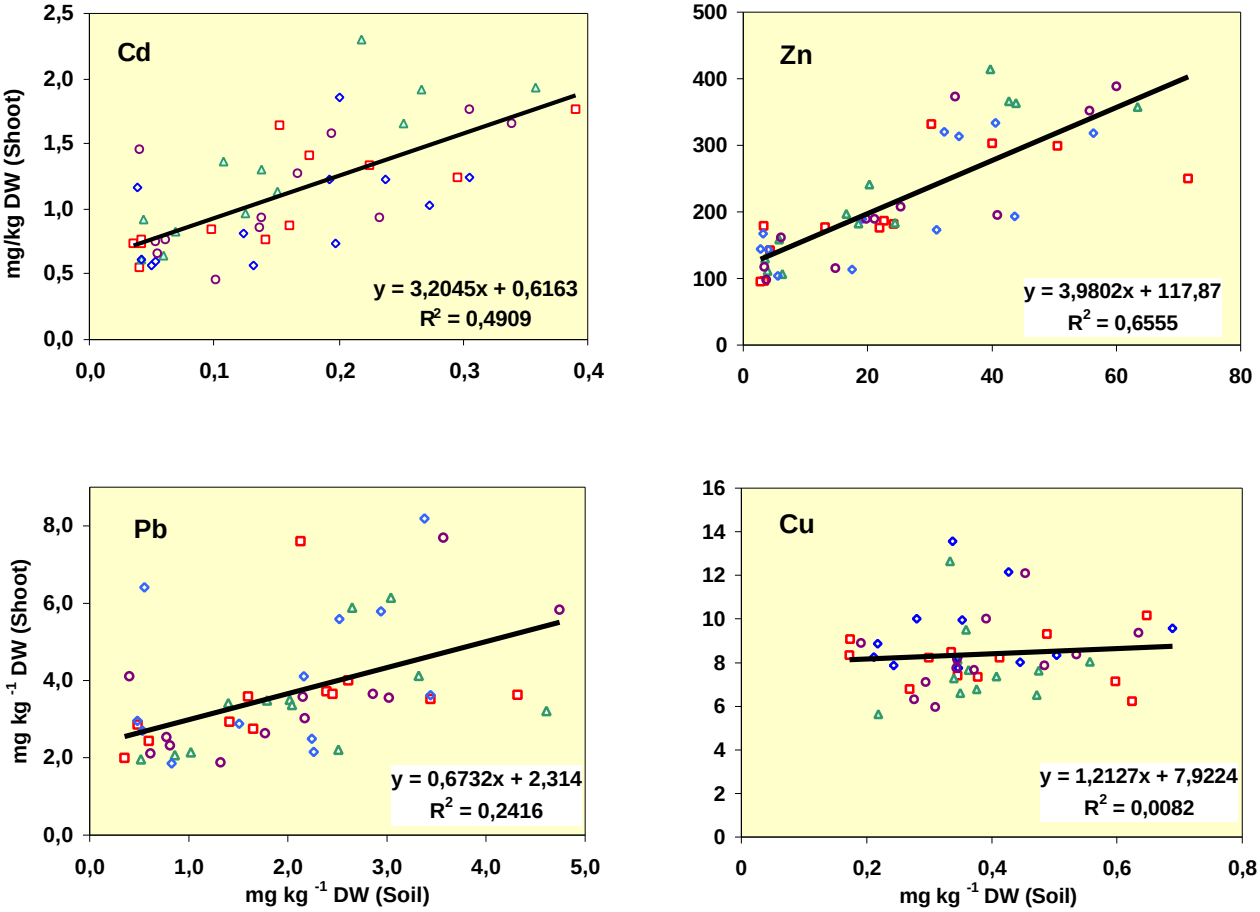


Figure 1: Relationship as determined by twofactorial linear regression between Cd, Zn, Pb and Cu in head lettuce and the ammonium nitrate extractable fraction of these elements in the three soils employed in the field experiments.(Cultivar identification: Mirian, squares; Martina, triangles; LM 8015, rhombs; Floret, circles).

Pot experiments

Total Cd, Zn, Pb and Cu content of the soils used in these experiments are shown in table 3. They are in the same order of magnitude if compared to the soils used in the field experiments. Without slag the content of the four elements was similar in both soils. Although the slag amendment and thus the heavy metal addition was the same, a somewhat lower content of these elements was measured in the slag treatments of soil A as compared to soil B, however.

The results of the ammonium nitrate and CAT extraction trials are given in table 4. Ammonium nitrate extractable Cd, Zn and Pb increased with increasing slag amendment, however, this increase was more pronounced at the lower pH level (Table 4). Around pH 5 Cd rose from about 0.03 to 0.4 mg kg^{-1} in the lighter soil A and to about 0.8 mg in the heavier

soil B, whereas around pH 7 a maximum of only 0.06 mg was found. Without slag, extractable Zn was about 1 - 2 mg kg^{-1} in both soils and pH levels. In the slag treatments it increased to about 100 mg around pH 5 whereas it reaches only 3 - 5 mg at the higher pH. Pb was low in all treatments without slag but with increasing slag amendment it amounted to about 5 - 13 mg kg^{-1} at the lower pH while only about 1 mg was measured at the higher end. This accounted for 4 – 12 % of total with Cd, 1 – 10 % with Zn and 1 – 2 % with Pb around pH 5 while at the higher pH this percentage was about an order of magnitude lower. Extractable Cu was about 0.1 – 0.3 mg kg^{-1} in all treatments accounting for approximately 0.5 - 1 % of total. As can be seen from the Cu data, extractability of this element responded only sligthly to the varied pH.
CAT extracted considerably more of the elements under investigation than did ammonium nitrate, yielding between 20 and 40 % of total Cd at both pH levels. Up to 30 % of total Zn was extracted around pH 5 and up to 20 % around pH 7. Pb recovery reached roughly 30 – 40 % of total at either pH levels. With Cu also 30 – 40 % of total was extracted around pH 5 while it still amounted to about 25 % at the higher pH.

At the lower pH Cd uptake into spinach shoots rose from about 2 mg kg^{-1} DW on uncontaminated soils to more than 20 mg on soil A and more than 40 mg at soil B due to the slag treatment. Zn uptake increased from 100 to 200 mg kg^{-1} DW to more than 3000 mg on soil A while the corresponding figures for soil B were 1200 and 1600 mg. Pb was detected in low but measurable concentrations in the shoot material. In the 10 % slag treatments it amounted to roughly 30 mg on soil A while a maximum of only 10 mg was found on soil B. At the higher pH level endogenous concentration of Cd, Zn and Pb were about one order of magnitude lower, which is also reflected in the reduction of the ammonium nitrate extractable portion of these elements at high pH. Cu concentration was in the order of 10 to 18 mg kg^{-1} DW, as found for bean leaves in the field experiment, and did not vary significantly irrespective of the treatments.
Shoot dry weight (DW) production in the various treatments are compared as a percentage of maximum yield on soil A or B, respectively (Table 5). On the lighter soil A top yield representing 100 % was obtained at pH level 7 without slag (A 7/0). At this pH dry weight production in the slag treatments was only sligthy reduced while no abnormal metal concentration was measured in the shoots. At the lower pH level dry weight yield was reduced by more than 80 %. For the slag free treatment (A 5/0) this cannot be explained by facts at hand since also no abnormal metal contents could be found. If slag was added to the soil, however, the depression of yield formation may be attributed to phytotoxic effects of high Cd and especially Zn concentration, the latter reaching a maximum of more than 3000 mg kg^{-1} DW at 10 % slag. Here not only growth reduction but also dying of more than 50 % of the plantlets shortly after emerging was observed. On soil B top yield formation (100 %) was attained at a pH around 7 and 5 % slag, with the other two treatments lagging only slightly behind. Different from soil A, only about a 30 % reduction of dry weight formation was noted on soil B without slag at the lower pH level (B 5/0). Here, the content of Cd and Zn in the shoot material reached the lower limits of potentially phytotoxic concentrations which, therefore, could have caused the growth retardation observed in this treatment. At this pH level the slag amendment caused the Cd and Zn concentration to rise to more than 40 and 1000 mg kg^{-1} DW, respectively, which may well be the reason for the yield depression of up to 90 %.

The relationship between the element fractions extractable from the soils with both extractants and the content of these elements in the spinach shoots was assessed by linear regression analysis, the results of which are depicted in figures 2 and 3. As can be seen, the correlation coefficients for Cd, Zn and Pb obtained with ammonium nitrate are better than 0.9 although

the slope of the regression line for Pb was much smaller than for the others. With CAT, r falls short of that, especially for Pb and it can also be seen that the slopes are, as a result of the higher extraction yield, inferior to those obtained with ammonium nitrate. As in the field trials no relationship could be established for Cu with either extractant. It should also be noted that ammonium nitrate extraction resulted in much better correlations for Cd, Zn and Pb than in the field experiments (Figure 1).

Table 3: Total heavy metal content of the soils used in the pot experiments. Data are mean ± SD (n = 5).

		Cd	Zn	Pb	Cu
Soil A	0 % slag	0.36 ± 0.03	66.6 ± 6.4	22.9 ± 1.5	15.0 ± 0.6
	5 % slag	2.61 ± 0.30	567,0 ± 80.2	288.9 ± 21.1	21.3 ± 1.8
	10 % slag	4.13 ± 0.27	1099,6 ± 25.9	522.9 ± 20.1	28.4 ± 1.7
Soil B	0 % slag	0.80 ± 0.16	64.3 ± 3.7	23.2 ± 1.7	17.2 ± 0.5
	5 % slag	4.61 ± 0.81	775.0 ± 85.2	300.9 ± 44.3	29.8 ± 3.6
	10 % slag	6.93 ± 1.96	1427.5 ± 241.2	665.2 ± 114,1	37.7 ± 4.6

Table 4: Heavy metals (mg kg^{-1}) extractable with 1 M ammonium nitrate or 0.01 M calcium chloride + 0.002 M DTPA (CAT) from different soils as dependend on pH level and slag amendment. Soil designation: A, loamy sand; B, silt loam; first numeral, pH level; second numeral, slag content (% w/w). For actual pH of the various treatments see table 5. (Data are mean ± SD, n = 5).

	Cd	Zn	Pb	Cu
Ammonium nitrate				
Soil A 5/ 0	0.03 ± 0.004	2.2 ± 0.5	0.18 ± 0.02	0.20 ± 0.02
A 5/ 5	0.25 ± 0.13	33.0 ± 9.4	2.93 ± 0.81	0.25 ± 0.07
A 5/10	0.42 ± 0.11	106.8 ± 19.8	12.91 ± 1.38	0.32 ± 0.11
A 7/ 0	0.01 ± 0.001	0.9 ± 0.2	0.08 ± 0.04	0.10 ± 0.01
A 7/ 5	0.02 ± 0.004	1.6 ± 0.4	0.48 ± 0.11	0.13 ± 0.01
A 7/10	0.02 ± 0.01	2.4 ± 4.3	1.02 ± 0.51	0.16 ± 0.02
Soil B 5/ 0	0.03 ± 0.01	1.2 ± 0.4	0.04 ± 0.01	0.07 ± 0.02
B 5/ 5	0.36 ± 0.04	47.7 ± 6.0	2.47 ± 0.46	0.15 ± 0.02
B 5/10	0.83 ± 0.17	83.7 ± 15.4	4.44 ± 0.85	0.26 ± 0.07
B 7/ 0	0.01 ± 0.001	0.6 ± 0.03	0.06 ± 0.01	0.06 ± 0.01
B 7/ 5	0.06 ± 0.03	3.3 ± 1.5	0.74 ± 0.33	0.11 ± 0.01
B 7/10	0.03 ± 0.01	3.7 ± 0.5	1.09 ± 0.54	0.12 ± 0.02
CAT				
Soil A 5/ 0	0.15 ± 0.002	11.4 ± 0.7	8.6 ± 0.3	7.27 ± 0.19
A 5/ 5	0.67 ± 0.07	136.3 ± 11.7	84.5 ± 4.1	8.01 ± 0.15
A 5/10	0.87 ± 0.07	235.2 ± 16.4	144.6 ± 3.2	8.21 ± 0.08
A 7/ 0	0.13 ± 0.004	14.6 ± 0.8	12.6 ± 0.3	5.37 ± 0.09
A 7/ 5	0.67 ± 0.05	83.2 ± 17.6	98.1 ± 7.0	5.68 ± 0.48
A 7/10	1.06 ± 0.05	127.2 ± 6.5	164.9 ± 6.7	6.01 ± 0.10
B 5/ 0	0.17 ± 0.02	11.0 ± 2.4	7.9 ± 1.2	5.07 ± 0.51
B 5/ 5	1.89 ± 0.12	236.2 ± 10.3	125.5 ± 2.6	9.37 ± 0.30
B 5/10	2.70 ± 0.10	313.8 ± 15.7	155.8 ± 13.5	12.32 ± 0.26
B 7/ 0	0.14 ± 0.01	12.2 ± 1.4	9.2 ± 0.4	5.21 ± 0.23
B 7/ 5	1.52 ± 0.66	164.0 ± 52.4	141.9 ± 28.5	7.66 ± 0.95
B 7/10	1.27 ± 0.10	153.8 ± 11.1	201.8 ± 17.7	6.52 ± 0.13

Table 5: Heavy metal content (mg kg^{-1} DW) and relative dry weight production of spinach shoots grown on different soils at varied pH and slag treatments. Relative dry weight production (Rel. DW prod.) was based on treatments with highest yield formation (100 %) on soil A or B, respectively (Soil designation as in table 4). Data are mean ± SD (n = 5).

	Actual pH	Cd	Zn	Pb	Cu	Rel. DW prod. (%)
Soil A 5/ 0	5.6 ± 0.2	1.59 ± 0.14	133 ± 9	0.54 ± 0.17	12.0 ± 1.8	16
A 5/ 5	5.1 ± 0.1	14.84 ± 4.40	1433 ± 288	3.23 ± 1.06	14.8 ± 3.9	16
A 5/10	4,9 ± 0.1	21.66 ± 0.36	3292 ± 9	29.01 ± 1.71	18.5 ± 0.2	7
A 7/ 0	6.9 ± 0.1	1.23 ± 0.07	91 ± 5	0.28 ± 0.12	11.4 ± 0.6	100
A 7/ 5	7.6 ± 0.4	2.24 ± 0.23	117 ± 6	0.93 ± 0.18	11.6 ± 0.8	87
A 7/10	8.0 ± 0.4	2.75 ± 0.17	142 ± 7	1.91 ± 0.40	11.8 ± 0.7	82
Soil B 5/ 0	5.6 ± 0.3	2.42 ± 0.54	181 ± 29	0.23 ± 0.04	14.0 ± 1.1	68
B 5/ 5	5.5 ± 0.1	28.74 ± 4.18	1228 ± 103	2.99 ± 1.06	10.8 ± 1.2	18
B 5/10	5.2 ± 0.4	44.01 ± 5.98	1608 ± 158	9.17 ± 0.87	11.6 ± 0.5	8
B 7/ 0	6.7 ± 0.4	0.99 ± 0.08	107 ± 6	0.25 ± 0.08	13.4 ± 0.7	84
B 7/ 5	7.4 ± 0.2	3.44 ± 1.24	192 ± 35	1.48 ± 0.82	12.6 ± 0.5	100
B 7/10	7.9 ± 0.1	2.84 ± 0.42	176 ± 30	1.50 ± 0.28	11.7 ± 1.0	95

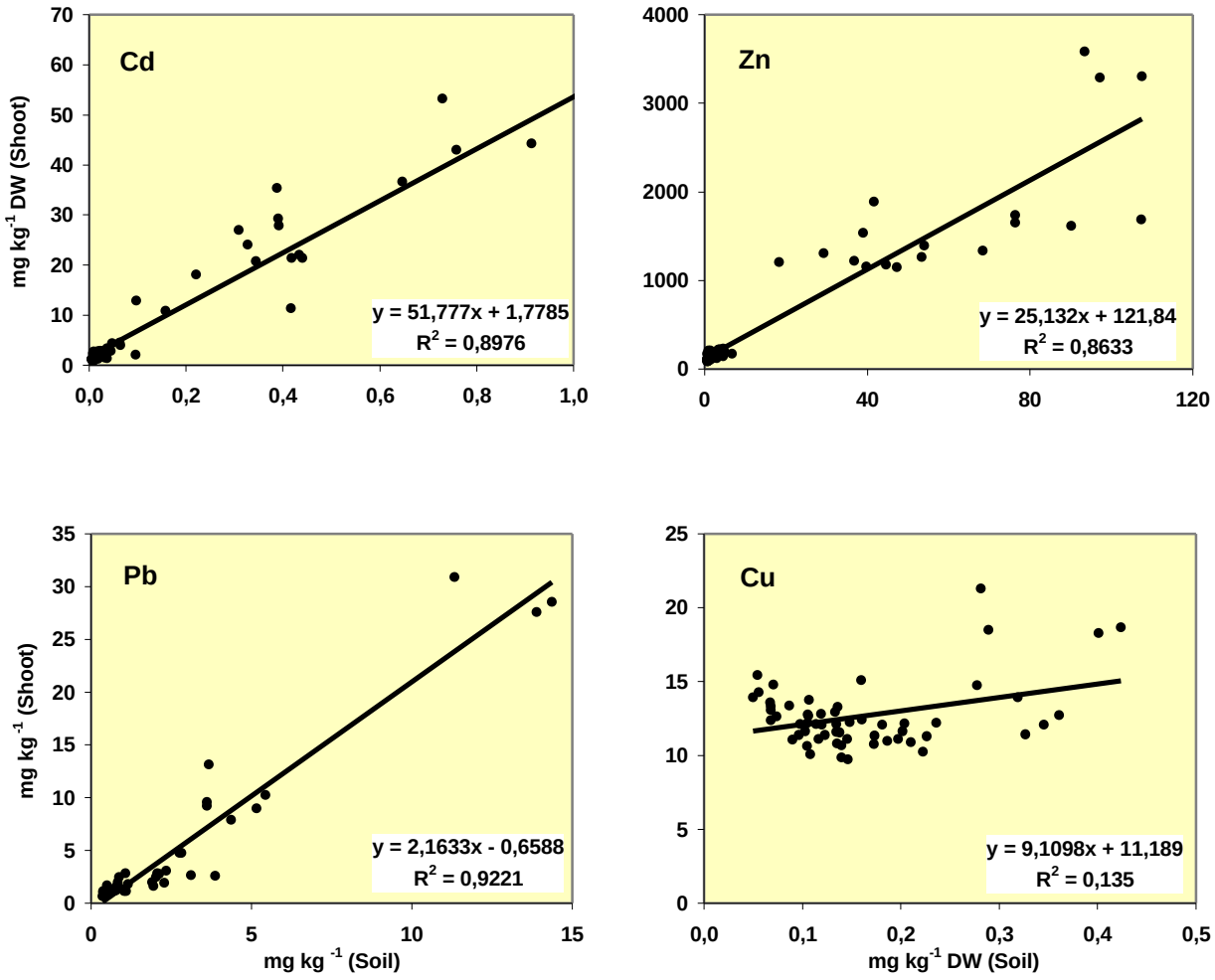


Figure 2: Relationship as determined by twofactorial linear regression between Cd, Zn, Pb and Cu in spinach shoots and the *ammonium nitrate* extractable fraction of these elements in two different soils (loamy sand, silt loam) containing 0 %, 5 % and 10 % (w/w) metallurgical slag at varied pH.

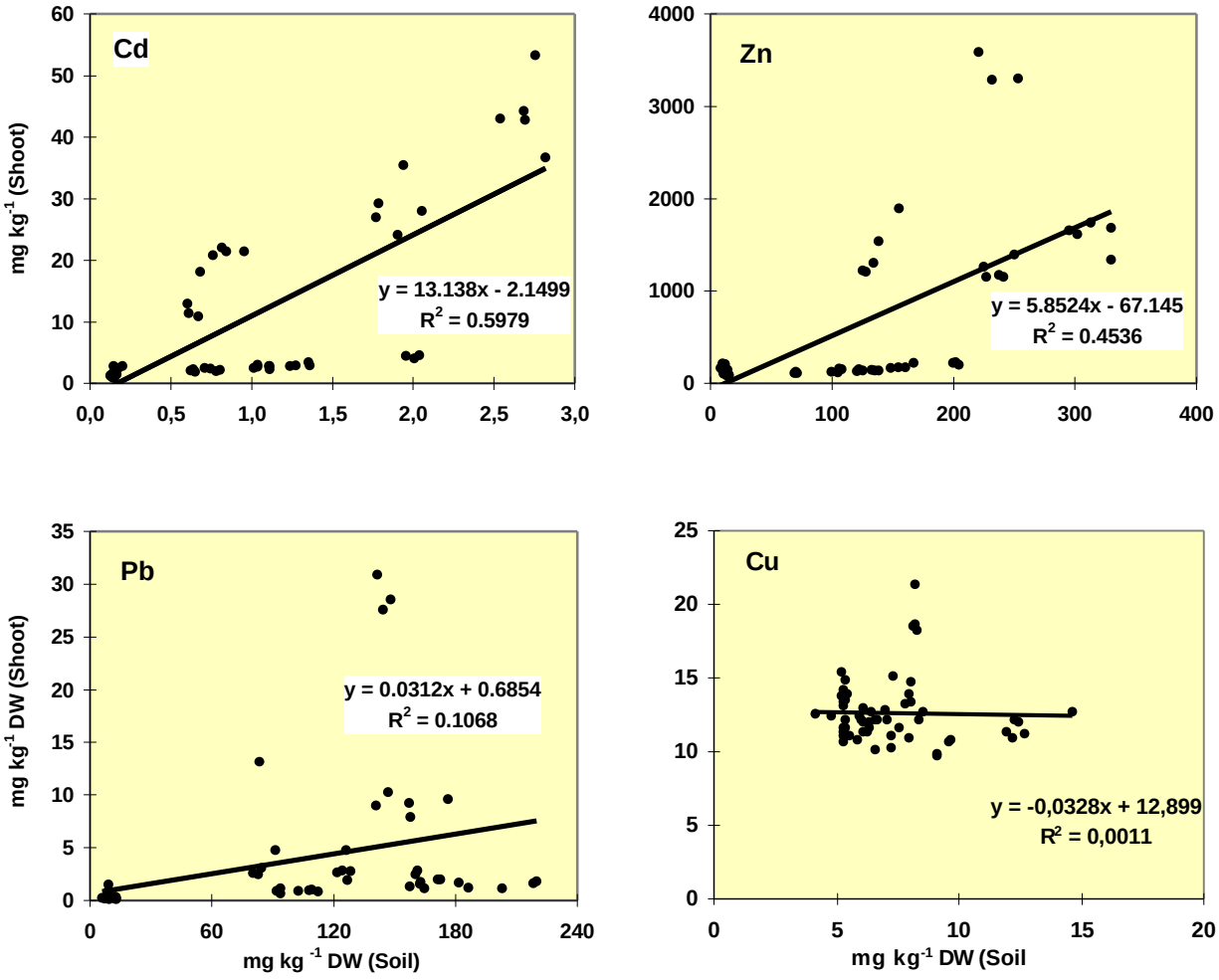


Figure 3: Relationship as determined by twofactorial linear regression between Cd, Zn, Pb and Cu in spinach shoots and the *CAT* extractable fraction of these elements in two different soils (loamy sand, silt loam) containing 0 %, 5 % and 10 % (w/w) metallurgical slag at varied pH .

Discussion

Problems caused by heavy metal contamination of arable soils include phytotoxic effects of certain elements such as Cd and Pb and also Zn and Cu which are well known as micronutrient elements, however can cause severe phytotoxicity if critical endogenous levels are exceeded (Mengel and Kirkby, 1982). Another and even more serious problem is posed by the uptake of potentially noxious elements by food or forage plant species and their transfer to the food chain and, finally, to humans (Kloke, 1980). Therefore, critical assesment of the phyto-availability of heavy metals in contaminated soils at pH levels falling within the range commonly found in arable soils is inevitable.
The determination of phytoavailable amounts of heavy metals in soils may be used to prognosticate their potential transfer to plants. Such information may in turn be helpful in deciding on the use of contaminated soils for the production of certain crops. This is

especially important if vegetables, which are directly consumed by humans, are to be grown on such soils.

In the field experiments it could be shown that Cd and Zn uptake was higher in lettuce and celery than in bush beans where the pods exhibited the lowest Cd concentration of all species and organs tested. Celery is known to take up heavy metals to a greater extent than do many other vegetable species. It was reported that celery leaves accumulate about 50 % more Cd in comparison to bulbs (Leh, 1988). This is not in accordance with our results as we measured about 30 % less Cd in the leaves than in the bulbs, however, it must be noted that Leh had found an order of magnitude higher Cd concentration in the celery material which may have caused different distribution pattern within the plant. Maximum levels of toxic heavy metals as recommended by german authorities, (BGVV, 1997) for edible plant parts (formerly known as ZEBS values) include Cd (0.1 mg kg^{-1} FW for lettuce and bean pods, 0.2 mg for celeriac bulbs) and Pb (0.8 mg kg FW^{-1} for lettuce, 0.25 mg for bean pods and celeriac bulbs). The Cd content of celeriac bulbs, if expressed on a fresh weight basis, exceeded the respective value on soil 2 and 3. Thus it must be concluded that the growing of celery cannot be recommended even on soils with moderate Cd contamination such as soil 3 and pH levels less than 6.5 to 6.8. Although the endogeneous Zn concentration of head lettuce and celery exceeded the critical level for phytotoxicity (150 to 200 mg kg^{-1} DW; Sauerbeck, 1982) about twofold on soil 3, no phytotoxic effects were noted. This implies a better than average Zn tolerance in these two species. Bush beans, on the other hand, did not accumulate critical amounts of Zn even on soil 3 indicating a better exclusion potential but also a lesser physiological requirement for Zn in comparison to the other species. Still it has to be taken into account that the given soil pH (6.3) does not favour Zn availability (Hornburg et al., 1995). Low Pb uptake by plants was expected at the given pH since the critical pH for increasing Pb availablility is well below 4 (Hornburg et al., 1995) and even celery did not exceed the assigned BGVV value. Cu content was lowest in celery leaves (5 to 7 mg kg^{-1} DW) while bulbs contained two to three times more. Head lettuce contained 30 to 50 % more Cu than celery leaves. In bean leaves a Cu content of 16 to 17 mg kg^{-1} DW was measured while pods showed only 2/3 of that concentration. These findings demonstrate nicely the different physiological Cu requirements of the three species and different organs of a given species. The observation that Cu uptake does not increase with soil contamination in either species can be explained if one looks at the ammonium nitrate extractable portion which also varies only insignificantly. Still, it remains unclear why this fraction did not increase with the increasing total Cu content.

Although cultivar dependend variations in heavy metal uptake of vegetable species have been reported (Lübben, 1991; Metz and Kloke, 1998) no significant differences between cultivars of either species were found in these studies. One of perhaps a variety of reasons may be that the soils were optimally fertilized and in particular rich in phosphorus (35 to 50 mg P kg^{-1}, CAL). In a recent paper it was demonstrated that differences between two spinach cultivars in the uptake of Cu, Zn and Cd were only found in phosphorus deficient plants (Keller and Römer, 2001) This implies that the nutritional status of plants should be considered in strategies aiming at the selection of genotypes with minimized uptake of potentially noxious heavy metals.

In the pot experiments the Cd and Zn content of the spinach shoots increased excessively at a pH level around 5 on both soils reaching more than 20 mg Cd and 3000 mg Zn on soil A 5/10 whereas more than 40 mg Cd and 1600 mg Zn were attained on soil B 5/10. The strong growth depression observed in these treatments was probably caused by the high endogeneous levels of Zn and also Cd since phytotoxic effects may be caused by the latter at plant levels

above 5 to 10 mg kg^{-1} DW (Sauerbeck, 1982), however, also Pb toxicity cannot be ruled out on soil A 5/10. The more than 80 % growth depression on soil A without slag (A 5/0), on the other hand, cannot be attributed to Cd and Zn toxicity as the endogeneous concentration of these elements did not reach critical levels. Moreover, in the corresponding treatment of soil B (B 5/0) only some 30 % dry weight reduction was noted although the shoots contained considerably more Cd and Zn. Here, the Zn content had just reached potentially phytotoxic levels so that the observed growth depression may partly be due to Zn toxicity. However, some other undefined factors must have also been involved as is indicated by the largely reduced growth on soil A 5/0. It is almost needless to say that in both slag treatments the spinach Cd levels exceeded the BGVV limits (0.5 mg kg^{-1} FW) up to four times. At the higher pH level the increase of the heavy metal content in the spinach shoots due to the slag amendments was negligible. As observed with the other species in the field experiments the Cu content of the spinach shoots varies only insignificantly. This is also reflected by rather small variations of ammonium nitrate and CAT extractable Cu.

The ammonium nitrate extractable fractions of Cd, Zn and Pb which decreased considerably with increasing pH correlated well with the content of these elements in the spinach shoots. Despite the much wider pH range correlation coefficients (r) were considerably better than in the field trials where only a very narrow pH range was tried. This implies that in pot experiments carried out in a more controlled greenhouse environment phytoavailability of the four elements tested can be assessed more precisely than in the field, however, it cannot be ruled out that the source of heavy metals, namely metallurgical slag as in these experiments, might influence chemical processes involved in extraction or element aquisition by plants. The high CAT extraction yield for all four elements even at the higher pH level may be a consequence of the low pH of this extractant itself and the added chelator DTPA. This extraction yield does not reflect the strongly reduced element uptake at this pH. Apparently, this is the reason why CAT soluble fractions of Cd, Zn and Pb did not correlate with uptake as good as did the ammonium nitrate soluble fraction. This is in accordance with results of Bucher and Schenk (1997) who found similar correlations employing the CAT method for the extraction of potentially phytoavailable Zn from peat substrates. While Alt and Peters (1993) reported good correlations for Cu using the CAT method on Cu deficient peat substrates, ammonium nitrate and CAT are apparrently not usefull for Cu in the soils and concentration ranges tested.

In summary it must be noted that transfer of potentially noxious heavy metals such as Cd or Zn to plants can occur in amounts that can adversely influence the nutritional value of food plants or cause yield depression even at comparably moderate soil contamination, especially if the soil pH is not strictly controlled. Ammonium nitrate extraction remains the method of choice if phytoavailable fractions of Cd and Zn are to be extracted in order to prognosticate potential transfer of these elements to plants while this method may or may not work with Pb. Still, the search for a suitable method which reliably extracts phytoavailable Cu from all soils and at all possible concentration ranges continues. Another main outcome of these studies is that heavy metal containing industrial slags which are frequently used in landscape construction or hydraulic engineering are not as chemically inert as reported (Lahl, 1994; Khorasani, 1999) if mixed with soils and thus being exposed to chemical processes occurring therein such as decreasing pH and others. Contamination of soils with such industrial waste products should, therefore, be avoided.

References

AbfallKlärV (1992): Klärschlammverordnung. BGBl I Nr. 21.

Alt, D. and I. Peters (1993): Die $CaCl_2$/DTPA-Methode zur Untersuchung gärtnerischer Erden auf Mengen- und Spurenelemente. 2. Mitteilung: Kupfer und Mangan. Agribiol. Res. 45, 215-224.

Alt, D., I. Peters and S. Gaus (1994): Die $CaCl_2$-Methode zur Untersuchung gärtnerischer Erden auf Mengen- und Spurenelemente. 7. Mitteilung: Zink. Agribiol. Res. 47, 75-83.

BGVV (1997): Richtwerte für Schadstoffe in Lebensmitteln. Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (ed.). Bundesgesundheitsblatt 40, 182-184.

Birke, C. and W. Werner (1991): Eignung chemischer Bodenextraktionsverfahren zur Prognose der Schwermetallgehalte in Pflanzen. In: Auswirkungen von Siedlungsabfällen auf Böden, Bodenorganismen und Pflanzen. Forschungszentrum Jülich (ed.), Berichte aus der ökologischen Forschung 6, 224-289.

Brümmer, G. W., J. Gerth and U. Herms (1986): Heavy metal species, mobility and availability in soils. Z. Pflanzenernähr. Bodenkd. 149, 382-398.

Brümmer, G. W. and V. Hornburg (1989): Untersuchungen zur Verfügbarkeit von Schwermetallen in Böden. Mitteilgn. Dtsch. Bodenkundl. Ges. 59, 727-732.

Bucher, A. and M. K. Schenk (1997): Characterization of phytoavailable zinc in compost-peat substrates – Comparison of methods. Z. Pflanzenernähr. Bodenk. 160, 595-601

Delschen, T. and F. Rück (1997): Eckpunkte zur Gefahrenbeurteilung von schwermetallbelasteten Böden im Hinblick auf den Pfad Boden/Pflanze. Bodenschutz 4, 114-121.

Dües, G. (1989): Bindungsformen von Schwermetallen im Boden, dargestellt auf der Basis fraktionierter Extraktion. In: Behrens, D. & J. Wiesner (eds.) Beurteilung von Schwermetallkontaminationen in Böden. Dechema, Frankfurt a. M., 143-167.

Hornburg, V., G. Welp and G. W. Brümmer (1995): Verhalten von Schwermetallen in Böden. 2. Extraktion mobiler Schwermetalle mittels $CaCl_2$ und NH_4NO_3. Z. Pflanzenernähr. Bodenk. 158, 137-145.

Kloke, A. (1980): Richtwerte `80: Orientierungsdaten für tolerierbare Gesamtgehalte einiger Elemente in Kulturböden. VDLUFA-Mitteilungen 1-3, 9-11

Kloke, A. (1988): Das „Drei-Bereiche-System“ für die Bewertung von Böden mit Schadstoffbelastung. VDLUFA-Schriftenreihe, Kongressband 28, 1117-1127.

Köster,W. and D. Merkel (1982): Beziehungen zwischen den Gehalten an Zink, Cadmium, Blei und Kupfer in Böden und Pflanzen bei Anwendung unterschiedlicher Bodenuntersuchungsmethoden. Landwirtsch. Forsch., Sh. 39, 245-254.

Khorasani, R. (1999): Die Beurteilung der Verwitterungsbeständigkeit und Umweltverträglichkeit von metallurgischen Schlacken. In: Schlacken in der Metallurgie. Gesellschaft für Bergbau, Metallurgie, Rohstoff- und Umwelttechnik (ed.) GDMD-Schriftenreihe 83, 237-249.

Lahl, U. (1994): Schlackenaufbereitung durch Alterung und Laugung. Müll und Abfall 31, 86-92.

Leh, H.-O. (1988): Schwermetallgehalte verschiedener Gemüsepflanzen und Möglichkeiten zu deren Verminderung durch ackerbauliche Maßnahmen – Teilergebnisse aus Freilandversuchen. Nachrichtenbl. Deut. Pflanzenschutzd. (Braunschweig) 40, 106-112.

Lübben, S. (1991): Sortenbedingte Unterschiede bei der Aufnahme von Schwermetallen durch verschiedene Gemüsepflanzen. VDLUFA-Schriftenreihe, Kongressband 33, 605-612.

Metz, R. and A. Kloke (1998): Einfluß der Sortenwahl auf den Cadmiumtransfer Boden-Pflanze. VDLUFA-Schriftenreihe, Kongressband 49, 139-142.

Mengel, K. and E. A. Kirkby (1982): Principles of Plant Nutrition (3rd Edition). International Potash Institute, Bern, Switzerland.

Sauerbeck, D. (1982): Welche Schwermetallgehalte in Pflanzen dürfen nicht überschritten werden, um Wachstumsbeeinträchtigungen zu vermeiden. Landwirtsch. Forsch. Sh. 39, 108-129.

Traulsen, B.-D. and G. Schönhard (1995): Verfügbarkeitsstudien über Schwermetalle und Nährstoffe im Boden. In: Folgenabschätzung in der Landnutzungsforschung. Werner, A. & M. Glemnitz (eds.). ZALF-Berichte 22, 20-24.

Preservation of Old Potato Varieties

Angelika Schäfer-Menuhr

Bundesforschungsanstalt für Landwirtschaft (FAL), Bundesallee 50,

38116 Braunschweig, Germany.

What are old potato varieties?

A potato variety may be defined as a group of identical plants, sharing the distinctive characters of an original individual from whom they are derived by vegetative reproduction. A potato variety is considered distinct when it differs from all other known varieties by one or more recognizable characters whether they be of a morphological or of a physiological nature. Commercial potato cultivars are propagated through the tubers, since the propagation by seeds is hindered by a high degree of heterozygocity, selfincompatibility and male sterility, frequently accompanied by failures in fruit and seed set.

In Germany for the commercialization of seeds of agricultural species and vegetables the addition to the National List is necessary. The German Seed Act (Rutz, 1999) provides the legal frame for the National List which protects the consumer and ensures the provision of high quality seed and planting stock material of resistant and high performance varieties for farmers and horticulturists. When potato varieties are no longer on the official list they are considered as old potato varieties.

Why does anyone want to preserve old potato varieties?

As long as varieties are still on the market the breeders maintain the varieties. But what happens with those varieties not any longer on the National List? They find a refuge in collections of plant genetic resources also known as genebanks, otherwise they could be lost for ever due to their requirements for vegetative propagation.

Tab. 1: Options for the long-term preservation of old potato varieties

Field genebank	**In vitro storage**	**Cryopreservation**
Long-term experience with the technique		
yes	yes	no
Availability for distribution		
several months of the year	always	3 to 6 months after thawing
Easiness of delivery		
high, with restrictions	high	low in N_2, high as plants
Feasibility to verify the genetic integrity directly		
high	low	low
Chance to keep cured material pathogen free		
non	high	high
Risk to lose part of the collection through diseases, pests etc.		
yes	non	non
Risk to lose part of the collection through technical failures		
high	high	non
Risk for mislabeling		
high	high	only in the initial phase
Costs for initiating the collection		
depending on circumstances	medium	medium
Costs for maintaining the collection		
depending on circumstances	medium	low

Though originally conserved for plant breeding purposes they are hardly if at all requested for this purpose. Requests come mainly from research institutions. Since a few years there is also a small but growing interest in old potato varieties among the consumers. However, the market share is very low. Mass commercialization is prohibited through the German Seed Act.

Fig. 1a: Storage as in vitro plantlets

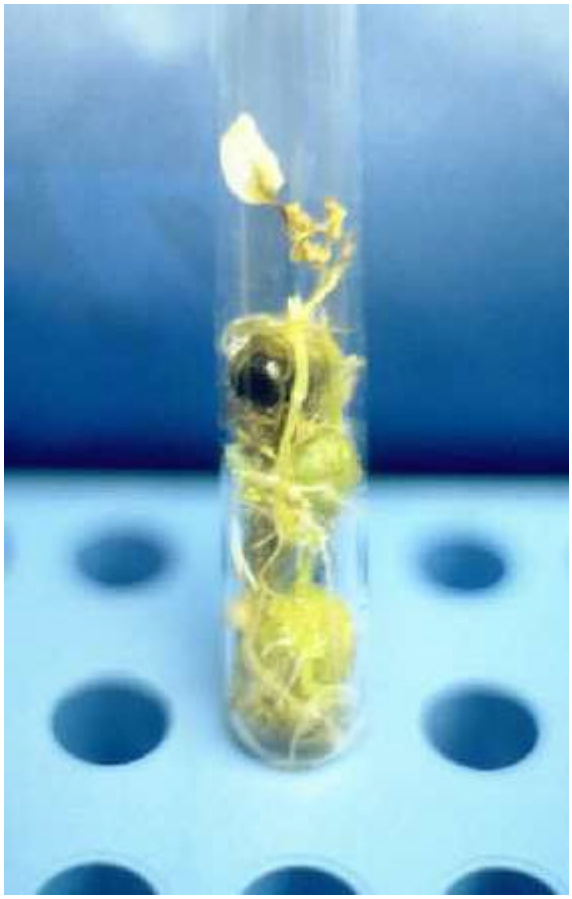


Fig. 1b: Storage as micro tubers

How can they be preserved?

Traditionally, potato varieties have been and are still maintained in a field genebank. The major advantage of growing the varieties in the field is the potential to verify the genetic integrity of the variety directly. Distribution of material is possible for several months at the time of the year seed potatoes are traditionally requested. The major disadvantage of a field genebank, however, is the risk to lose part of the collection through diseases, pests, weather damage or other accidents. In Tab. 1 the second option for the long-term storage of potato varieties is the in vitro storage, either as plantlets (Fig. 1a) or as microtubers (Fig. 1b). This type of storage lowers the risk to lose material due to environmental stress. Once viruses or other pathogens have been eliminated cultures can be kept pathogen free. But the in vitro storage is not totally secure, potential risks are technical failures, secondary infection or mis-

labeling. The third option is cryoconservation, the storage in liquid nitrogen. Also this method has advantages and disadvantages. From the three options for long-term conservation of vegetatively propagated potato genotypes, field genebanks have the longest tradition, also in vitro storage techniques exhibit long-term experience whereas cryopreservation of potato as a routine storage method is fairly new.

Cryopreservation - a method for long-term storage?

First successful attempts to freeze meristems or shoot-tips of Solanum tuberosum genotypes were already published 1978 by Bajaj followed by a number of publications (see Schäfer-Menuhr et al., 1996 for references). Though the published methods were partially successful they have never been refined further for routine application in a collection of plant genetic resources. Thanks to the initiative of Lindsey L. Withers, a joint project between the Federal Agricultural Research Centre (FAL) and the German Collection of Microorganisms and Cell Cultures (DSMZ) supported by the Minister for Economic Cooperation through the International Plant Genetic Resources Institute (IPGRI) was originated to explore already existing freezing methods, select a promising one, refine it for a genebank routine and finally apply it. As scientist in charge I want to introduce the method and give an overview about my experiences using this method in the routine of both German collections of plant genetic resources, one of which still situated in Braunschweig and the other one in Gatersleben.

The published freezing protocols vary and are mostly the result of trial and error rather than a complete understanding of the freezing process. From the already existing freezing methods I selected ultra rapid freezing. With this freezing technique the intracellular ice crystallizes in microcrystals of a size which is supposed to be harmless to the integrity of the cell components. Then I had to decide on the plant material to use and a way enabling not only ultra rapid freezing, but also ultra rapid thawing which I think is also necessary and, thirdly, I needed a way to store the material in liquid nitrogen. The plant material supposed to be cryopreserved was stored under slow growth conditions. Comparative growth experiments led me to the decision that the plantlets should be grown in fairly high density with good airation. Then I had to decide on the explants and their size. For ultra rapid freezing they had to be small, on the other hand small meristems of the size of those prepared for virus elimination were time consuming to prepare and had already a poor regrowth rate when not frozen. A good compromise were trimmed shoot tips.

Fig. 2a: Ultrarapid freezing

Fig. 2b: Storage in liquid nitrogen

The second problem was to find a kind of support for the shoot-tips that allows not only ultra rapid freezing but also ultra rapid thawing and that can be stored in liquid nitrogen containers in a way that they can be found again when needed. I am very much indepted to Erica Benson who introduced me to the idea to use small aluminum foils (described by Kartha et al., 1982) fitting into the standard 2 ml cryovials which can be labeled and stored in a documented place in the cryocontainer.

With these two fixed parameters (trimmed shoot-tips and aluminum foils as support) I started to optimize the method. Most experiments were performed with several varieties. The result was a simple, almost primitive, freezing method which is briefly described in the following (for details see Schäfer-Menuhr et al., 1996). The varieties to be frozen are propagated in high density (10-15 in vitro plantlets) in vessels allowing good air exchange. These are 12 cm high twist off jars with a hole in the lid into which a cotton plug had been inserted. Depending on the variety, it takes 3 to 7 weeks until the plantlets have reached a height of 10 cm. Then the shoot-tips are cut off, trimmed under a stereomicroscope in a way that they contain the apical meristem, leaf primordia and part of the covering leaves. The actual size varies depending on the in vitro growth habit of the variety. For one freezing experiment 100-150 shoot-tips are prepared and incubated in MsTo-medium (Towill, 1983) over night at 23 °C. The next day the shoot-tips are transferred into the cryoprotectant which is the same medium containing 10% dimethylsulfoxide. After incubation for 2 hrs (actually it is a broad plateau of 1-4 hrs) the shoot-tips are placed into small droplets (2.5 µl) of cryoprotectant which had been distributed

on small rectangles of aluminum foil (Fig. 2a). The foils are inserted into labeled cryotubes which had been filled with liquid nitrogen. The tubes are closed and placed into the storage container (Fig. 2b). Directly after freezing one vial containing 10-12 shoot-tips is checked for its ability to regenerate plants. We froze every variety three times which means that about 20-30 cryovials are stored per variety.

Though the method is comparably fast, there are, without doubt, limitations concerning the number of samples that can be frozen. A limiting factor is the overnight incubation which is absolutely necessary for the success of freezing. This means freezing is reduced to 4 days of the week or even reduced to two days per week if there is a legal holiday in the middle of the week. If one technician does everything she/he can freeze 50 varieties per year which means 10 years for 500 varieties. The freezing efficiency can be raised considerably if two persons are available for the freezing work. It´s quite realistic to assume that they can freeze 150 varieties per year. This would reduce the time for freezing 500 varieties to 3-4 years.

During the five year period of the project Ellruth Müller and I froze 219 varieties and genotypes. Theoretically, the two of us could have frozen 500 varieties or more, if we would not have had to deal with other tasks like finding a suitable method, optimizing the technique, verifying the genetic stability and last but not least writing reports and publications. Our inheritance to Gunda Mix-Wagner who continued the work at the genebank in Braunschweig later on, was a simple freezing method, a post freezing regeneration system which certainly works at a suboptimal level for several genotypes, but has the advantage that plants have been obtained from all varieties frozen so far, and two liquid nitrogen containers with 63 852 shoot-tips in 5 321 cryovials.

Of every batch frozen 10-12 shoot-tips have been thawed to check the ability for plant regeneration after freezing. These regrowth controls were used to calculate the survival and plant regeneration rates. Since only 10-12 shoot-tips were thawed for the regrowth controls (for the first varieties frozen it were only 5-6), the calculations are more approximations than statistically secured data. In addition, neither the regeneration medium nor the culture conditions are optimal for all genotypes. The survival rates for most varieties are high - approximately 80 % on the average. More important for a genebank, however, are the plant regeneration rates. They are, on the average, 40 % (40 % means in practice 4 plantlets per 10 frozen shoot-tips) and depend on the genotype. This genotypic effect is well known to anyone working with in vitro cultures of potato. As an example, the regeneration ability of the first 200 varieties frozen are shown in Fig. 3. The columns represent percentage groups. I think it is safe to assume that a plant regeneration rate of 30 % is not only sufficient for the maintenance of the variety but also for a potential distribution. Fig. 3 shows clearly that this is the case for 129 varieties. It is now a question of time and confidence in the method what to do with the 71 varieties having plant regeneration rates below 30 %. Since they have also proved that they regenerate plants, enough vials have been stored. Thus, shoot-tips of several vials can be thawed in case the variety should be requested. The fact that plants have been obtained from all 219 varieties which had not been especially selected for the freezing proceedure presented in this paper makes this method attractive for collections of plant genetic resources.

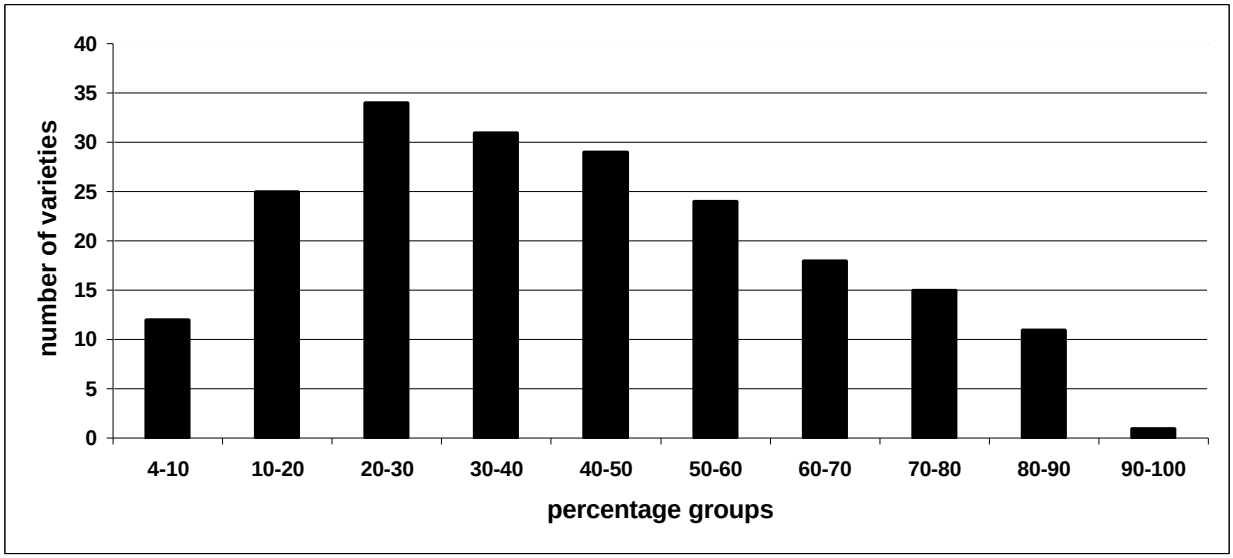


Fig. 3: Plant regeneration of the first 200 varieties frozen

An important criterion whether or not this freezing method is useful for the long-term storage of potato varieties is the question whether the genetic characteristics of the varieties or genotypes are maintained. It is a matter of definition how narrow or how wide the term "genetic identity" is defined. A 100% congruence is difficult to prove and is also not reasonable. For a practical application it should be sifficient to show that the characteristic attributes of a variety are conserved for a potential use in future breeding programs.

The most effective method to show that the freezing and regeneration process did not change the properties of a variety was the evaluation of the phenotype of regrown plants. Among the regrown plants of 98 varieties only one abnormal plant was found which was most likely polyploid. There were also a few weak or tiny plants with poor or no tuber production. I do not think that the reduced vitality had anything to do with the freezing process, because we observed it also among the control plants. Similar phenomenons have been described in the literature (Lommen 1996a, 1996b). Besides these few exceptions the regrown plants grew vigorously and could not be distinguished from the control plants.

The other two methods, flow cytometric measurements and DNA-fingerprinting, respectively, were less effective tools to prove the genetic identity. Though flow cytometry is an easy method for the determination of ploidy levels, the results are difficult to interpret, if the analyzed material is not in the same physiological state. However, the results showed clearly that no polyploid specimen was among the analyzed plants.

Employing DNA-fingerprinting we did not find unusual banding patterns, but this does not mean anything. Variants can only be detected, if the mutation is within the cleavage site of the respective restriction enzyme. In addition to that, it is very cumbersome to distinguish between real mutations and artifacts which occur when impurities in the extract disguise the cleavage site. In my opinion variation or somaclonal variation are no problem when using cryopreservation for long-term storage. The frequency is certainly not higher than in a field genebank or in in vitro storage.

Almost five years of using this simple freezing technique in the routine of a collection of plant genetic resources has shown that this method allows the secure storage of potato varieties and genotypes. The most important factors for the regeneration of plants after freezing are the preculture of donor plants and the postculture after freezing.

After termination of the initial project freezing was gradually continued in the FAL, however, initially at a reduced rate due to a reduction of staff. Activities increased considerably later on within the frame of the EU project “Genetic resources of potato including conservation, characterization and utilization of secondary potato varieties for ecological production systems in Europe”. In 1997 cryopreservation of potato was also started at the Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben using the same freezing protocol but micro tubers (Fig. 1b) as starting material. The growth controls in both institutes show that survival and plant regeneration is in about the same order of magnitude as was previously found by us.

Perspectives

When reflecting about the years, cryopreservation of potato is carried out and in the discussions pro and con cryopreservation one question that seems to be completely unimportant to those making final decisions in research policies, is the fate of the cryopreserved potatoes in the far future. We are very confident that the samples will still have the same regeneration behavior after many years and theoretically the samples can be stored forever and will not lose their vitality as long as liquid nitrogen is refilled. The problem is that the know-how is lost very fast when the freezing activities are interrupted for a longer time in spite of the simplicity of the method and an easy to follow manual. The danger increases when all samples are put into storage and when they are no longer integrated in an in vitro collection. Without in vitro culture techniques the samples cannot be thawed and regenerated. Therefore it is quite clear that cryopreservation as sole long-term storage technique cannot substitute in vitro culture collections or field genebanks, if old potato varieties are to be conserved for future generations.

Acknowledgements

Part of this work has been supported by the Minister for Economic Cooperation through the International Plant Genetic Resources Institute (IPGRI) and the Commission of the European Communities, Directorate-General for Agriculture, DGVI (RESGEN). The excellent technical assistance of Ms. Ellruth Müller is greatly acknowledged.

References

Bajaj, Y.P.S. (1978): Tuberization in potato plants regenerated from freeze-preserved meristems. Crop Improvement 5, 137.

Kartha, K.K., N.L. Leung and L.A. Mroginsky (1982): In vitro growth responses and plant regeneration from cryopreserved meristems of cassava (Manihot esculenta Crantz). Zeitschrift für Pflanzenphysiologie 107, 133-140.

Lommen, E.J.M. (1996a): Variation between individual plants in potato crops as affected by the weight of the seed tuber. pp.112-113 in Abstracts, 13th Triennial Conference of the European Association for Potato Research. Veldhoven, The Netherlands, 14-19 July, 1996.

Lommen, E.J.M. (1996b): Improving the field performance of in vitro produced plantlets of early cultivars. Ibid, pp. 114-115.

Rutz, H.-W. (Ed.) (1998): Sorten- und Saatgut-Recht. 8. Auflage. Agrimedia.

Schäfer-Menuhr, A., E. Müller and G. Mix-Wagner (1996): Cryopreservation: an alternative for the long-term storage of old potato varieties. Potato Research 39, 507-513.

Towill, L.E. (1983): Improved survival after cryogenic exposure of shoot tips derived from in vitro plantlet cultures of potato. Cryobiology 20, 567-573.

Factors Affecting Androgenesis in Indica Rice

Sant S. Bhojwani, Himani Pande and Anupam Raina

Department of Botany, University of Delhi, Delhi 110007, India
e-mail: ssbhojwani@satyam.net.in

Abstract

For its effective utilization in breeding programmes, the haploid production technique should allow genotype-independent production of large numbers of haploids. Although anther culture has been successfully used to hasten the breeding programmes in several crop species, including rice, there still remain problems to realize its full potential. Unlike the highly responsive model systems, most of the indica cultivars respond rather poorly in anther cultures. There is also concern regarding the gametic spectrum representation by the anther culture-derived doubled haploids. If there is a biased representation of recombinants possessing better culturability, it is important to analyse how seriously it effects the objectives of the breeder.

Whereas in maize androgenesis occurs via embryogenesis, in barley, wheat and rice the microspores divide to form a callus which later differentiate plants. Both the steps require different culture conditions and are affected by the genetic make-up of the plants. Recently, the physiology of the donor plants has been identified as a critical factor in achieving better anther culture efficiency. In this paper the factors affecting androgenesis in rice, particularly indica rice will be discussed.

Genotype

Most of the *in vitro* morphogenic responses are genotype–dependent (Bhojwani and Razdan, 1996). In general, indica cultivars of rice exhibit poorer androgenic response than the japonica cultivars (Hu, 1985, Raina, 1997). Miah *et al*. (1985) reported that anther culture response varied from 41 % for a japonica cultivar to 0 % for an indica cultivar. Even among the indica cultivars a considerable variation for pollen callusing and plant regeneration has been observed. Guha-Mukherjee (1973) reported that only 5 out of 18 indica cultivars showed pollen callusing and in only four cases did the calli differentiate plants. Similarly, Lentini *et al*. (1995) reported that only one out of 35 indica cultivars exhibited pollen callusing on N_6 medium. The purple pigmented, coarse-grained indica cv Crossa performed significantly better (40 %) than the fine grained, aromatic cv Basmati-370 (<10 %; Raina, 1989).

Physiology of Donor Plants

The physiological state of the donor plants, which is affected by several factors, has a profound effect on the androgenic response of their microspores. In *Brassica juncea*, a recalcitrant species, we were able to improve the androgenic response from 3 % to 16 % only by late sowing of the donor plants, which probably acted as a stress on the plants (Agarwal and Bhojwani, 1993). Our more recent experience with this oilseed crop is that under controlled growth conditions, in a phytotron or growth chamber, the donor plants can be maintained for a long time without effecting the androgenic potential of their anthers, and the difference between the anthers from main branch and lateral branches of different order is considerably reduced. We do not know of any similar study with rice but there is sufficient evidence to suggest that the growth conditions of the donor plants have significant effect on the yield of androgenic pollen in this crop.

A remarkable effect on the androgenic response of an indica rice cultivar by light and day/night temperature regime was observed by Raina and Zapata (see Raina, 1997). The plants of cv IR43 that reached the panicle emergence stage under long days (>12 h), high solar radiation (>18Mj m^{-2}) and sunshine (>8 h) and day/night temperature (34 ^{o}C/24 ^{o}C) showed highest anther culture response; the response declined with lowering values of these parameters. They also observed that the plants grown in the field were significantly superior in this respect to those grown in the glasshouse or in pots near the field. Superiority of field-grown plants over glasshouse-grown plants has also been reported for other cereals, including maize (Petolino and Thompson, 1987) and wheat (Lu *et al*., 1991).

The physiology of the donor plants can also be altered, and the androgenic response modified, by treating the donor plants with certain chemicals, such as etherel (Zhao et al., 1991, *cited in* Sun, 1999).

Pre-Treatment

Application of a variety of stresses, such as temperature pre-treatment, osmotic shock and sugar starvation, during the labile developmental period of pollen grains is known to be promontory or essential for the induction of androgenesis in several plants, including cereals (Bhojwani and Razdan, 1996; Bhojwani *et al*., 1997). However, the type, duration and the time of application of these pre-treatments may vary with the species or even variety (Datta, 2001).

Low Temperature

The most widely used pre-treatment for androgenesis is the low temperature shock. In most of the published works on androgenesis in rice, panicles were given a cold pre-treatment but the temperature and duration varied. Matsushima *et al.* (1988) had reported that a pre-treatment at 10 ^{o}C for 10-30 days was necessary to induce sporophytic divisions in microspores of the japonica cv Nipponbare. This was subsequently confirmed by several workers for japonica and indica cultivars (Ogawa *et al*., 1992, Datta *et al*., 1990, Raina and Irfan, 1998). Ogawa *et al*. (1995) observed that 28 days of pre-treatment at 10 ^{o}C was optimum for the indica cv IR 24. Gupta and Borthakur (1987) had selected pre-treatment at 10 ^{o}C for 11 days for anther

culture of the indica cultivar khonorullo. Although the frequency of anthers showing pollen callusing after cold-treatment for 25 days was fairly high, most of the plants regenerated from the calli formed after such a long cold pre-treatment were albinos. Similarly, Pande (1997) observed that cold pre-treatment was essential for androgenesis in anther cultures of the indica cv IR43, and 10 ^{o}C for 10 days was most suitable. Pre-treatments longer than 11 days resulted in albino production. Reddy *et al.* (1985) reported that a brief (10 min) exposure to high temperature (35 ^{o}C) before cold-treatment was better for pollen callusing but it adversely affected green plant production.

Osmotic Stress

Another pre-treatment, which can substitute cold treatment for the induction of androgenesis, is osmotic shock. Wei *et al.* (1986), who first time reported *ab initio* microspore culture of barley, followed a protocol involving isolation of microspores in 0.3 M mannitol and treating them in this solution for 3 days. According to them, mannitol pre-treatment for 3 days was essential and for 7 days optimum to induce androgenesis in this system. Roberts-Oehlschlager and Dunwell (1990) had reported that 4 day incubation of barley anthers in a medium containing 3.2 % mannitol raised the pollen callusing response from 23 % to 78 % which was more than cold treatment alone or in combination with mannitol. According to these authors, mannitol stress improves sugar uptake causing build-up of glucose pool in the anther tissue. Similar results were reported by Ziauddin *et al.* (1990). Hoekstra *et al*. (1992) and Cistue *et al.* (1994) confirmed the importance of mannitol pre-treatment. In the latter case, application of 0.7 M mannitol for 3-5 days improved green plant regeneration significantly. Increase in mannitol concentration showed linear increase in the number of dividing microspores and an increase in the proportion of green plants regenerated. The optimum duration of treatment varied with the genotype. Hoekstra *et al*. (1993), who gave mannitol stress (440 mOs kg^{-1}) for 4 days and cultured the microspores on a medium of the osmolarity of 350 mos kg^{-1} at a density of $2x10^{4}$ microspores per ml, obtained 320 embryo-like structures per 1x104 microspores. The yield of green plants increased from 50 % to 97 %.
Recently, Raina and Irfan (1998) have reported that treatment of anthers in 0.4 M mannitol solution was essential to induce androgenesis in microspore cultures of indica and japonica cultivars. For indica cv, pre-treatment at 33 ^{o}C was better than at 25 ^{o}C, with respect to the number of embryo-like structures or calli formation. In the absence of cold treatment, mannitol treatment promoted androgenesis in anther cultures of cv IR43 from 3 % to 33.4 %; with cold treatment it had no promotory effect (Pande, 1997). For wheat microspore culture, Mejza *et al*. (1993) isolated and fractionated microspores in 20 % maltose solution and cultured them on a medium with 9 % maltose. This allowed genotype non-specific regeneration in *ab initio* microspore cultures. Cold treatment in combination with the osmotic treatment was detrimental. Therefore, maltose treatment was given at 25 ^{o}C.

Ogawa *et al*. (1995) reported that sugar starvation of microspores for 3 days could, to some extent, substitute for cold treatment for the induction of androgenesis in microspore cultures of indica rice. Sugar starvation of the anthers of IR43 for 2 days in the beginning of culture caused12-fold increase in the androgenenic response (39 %)of cv IR43 (Figure 1; Pande, 1997). However, cold treatment was superior to sugar starvation. Sugar starvation has been earlier shown to promote androgenesis in tobacco (Aruga *et al*., 1985) and barley (Wei *et al.*, 1986).

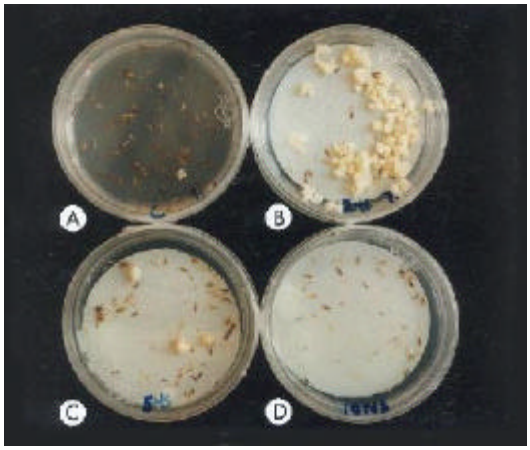


Figure1: **A-D**. Effect of sugar starvation for 0, 2, 5 and 10 days, respectively, on androgenic response in anther cultures of rice cv. IR43. Maximum pollen calli were formed in 2 days-starved cultures.

Culture Medium

Chu (1975) had demonstrated that the level of ammonium nitrogen in the culture medium is critical for androgenesis in rice. On this basis he developed the N_6 medium which has been most widely used for rice anther culture (Raina, 1989, 1997). However, this medium has proved significantly better than most other media for japonica rice. The indica cultivars require even lower NH_4^+ ions. Reddy *et al.* (1985), who studied 8 indica cultivars, found He_2 medium to be better than N_6 medium. He_2 medium is derived from N_6 medium by reducing NH_4^+ to half strength and $MgSO_4$ to $1/50^{th}$ level, and doubling the concentration of KH_2PO_4. In Korea N_6-Y1 medium was recommended for indica-japonica hybrids (Chung, 1987). This medium is essentially the same as N_6 medium except that $(NH_4)_2SO_4$ has been reduced from 3.5 mM to 1.5 mM.

In 1989, Raina reported that a medium with high KNO_3 and NH_4^+ ions completely replaced by 50 mg/l casein hydrolysate (CH) was significantly better than the original MSN medium for indica x indica F_1 hybrids, with regard to the frequency of green plant regeneration. This SK-1 medium was half as effective as N_6 medium so far as the frequency of pollen callusing is concerned but the calli formed on this medium produced twice as many green plants as those on N_6 medium. More recently, Raina and Zapata (1997), based on their detailed study on the medium requirement of the indica cultivar IR43 evolved a new medium called M-019 (*see* Raina and Irfan, 1998). This medium differs from SK-1 medium in substitution of CH by a small amount of $(NH_4)_2SO_4$ and the level of KH_2PO_4 raised from 170 mg/l to 540 mg/l. Ogawa *et al*. (1995) studied the effect of nitrogen source on androgenesis in another indica cultivar IR24, using R-2 medium as the control. R-2 has 40 mM KNO_3 and 2.5 mM $(NH_4)_2SO_4$. When 20 mM KNO_3 was combined with a small amount of $(NH4)_2SO_4$, glutamine or alanine all treatments induced pollen callusing but alanine was the best supplement. It not only induced high frequency androgenesis but also showed maximum regeneration of green plants.

Amino acids have been used as nitrogen source in *in vitro* culture of various tissues, including isolated microspores. Cho and Zapata (1988) reported that proline and glutamine promoted callus formation in microspore cultures of a japonica cultivar. These amino acids also induced a higher degree of plant regeneration and green plant production than the medium containing alanine or no amino acid. Ogawa *et al* (1995) also found glutamine to be

promotory for pollen callusing in microspore cultures of an indica cultivar but alanine was far better than glutamine for plant regeneration and green plant production.

Table 1: Composition of the culture media used for rice anther/microspore culture

Constituents	Media (mg l^{-1})			
	He_2*	MO19**	N_6***	C****
KNO_3	3181.5	3101.0	**2830.0**	3134.0
KH_2PO_4	800.0	540.0	**400.0**	540.0
$(NH4)_2SO_4$	231.0	264.0	**463.0**	231.5
$MgS0_4.7H_2O$	3.5	370.0	185.0	185.0
$CaCl_2.2H_2O$	166.0	**440.0**	166.0	150.0
KI	0.8	0.8	0.8	0.83
H_3BO_3	1.6	6.2	1.6	6.2
$MnSO_4.4H_2O$	4.4	22.3	4.4	22.3
$ZnSO_4.7H_2O$	1.5	8.6	1.5	8.6
$Na_2MoO_4.2H_2O$	-	0.25	-	0.25
$CuSO_4.5H_2O$	-	0.025	-	0.025
$CoCl_2.6H_2O$	-	0.025	-	0.025
$FeSO_4.7H_2O$	167.1	27.8	27.8	27.8
$Na_2EDTA.2H_2O$	223.5	37.3	37.3	37.3
Inositol	-	100.0	-	-
Nicotinic acid	2.5	2.5	0.5	2.5
Pyridoxine HCl	0.5	2.5	0.5	2.5
Thiamine HCl	0.5	1.0	1.0	2.5
Glycine	2.0	2.0	2.0	2.5
$AgNO_3$	-	-	-	10.0
Casein hydrolysate	250.0	-	-	-
Yeast extract	1000.0	-	-	-
Sucrose	30000.0	40000.0	40000.0	-
Maltose	-	-	-	50.0

* Huang et al. (1978)
** Raina & Irfan (1998)
*** Chu et al. (1975)
**** Letini et al. (1995)

Since 1983, maltose has been shown to be a superior source of carbohydrate than sucrose for androgenesis in several species, including cereals (Finnie *et al*. 1989; Last and Brettell, 1990; Pande and Bhojwani 1999). A significant increase in anther culture efficiency and green plant formation in otherwise highly recalcitrant indica rice cultivar occurred when sucrose was replaced by maltose (Raina, 1997).

Lentini *et al*. (1995) reported that on N_6 medium with 146 mM sucrose only one out of 23 indica cultivars exhibited pollen callusing and green plant production. Substitution of sucrose by equimolar amount of maltose enhanced pollen callusing from 6.3 % to 10.1 % and green plant regeneration from 0.6 % to 1 %. Glucose was inhibitory. Earlier, Mejza *et al*. (1993) had observed that isolation and fractionation of microspores in 20 % maltose solution, and their culture on a medium containing 9 % maltose allowed genotype-independent plant regeneration in *ab initio* microspore cultures of wheat. Maltose promoted the direct

embryogenic pathway of plant formation from pollen in wheat (Navarro-Alvarez *et al*., 1994), maize (McDonald, 1992) and barley (Powell *et al*., 1992).

In cultures, sucrose rapidly breaks down to glucose and fructose, so that after 3 weeks of barley anther culture the medium did not contain any sucrose (Last and Brettell, 1990). On the other hand, the hydrolysis of maltose during the same period was below the detectable level. The toxicity of sucrose for androgenesis is due to the sensitivity of microspores to fructose but not to glucose.

Calcium in the medium is known to stimulate ethylene production in many plant tissues. Addition of the calcium ionophore A23187 (0.5 μM) along with $CaCl_2$ (1 mM) enhanced pollen callusing over $CaCl_2$ alone. Addition of 10 mg L^{-1} of $AgNO_3$, an anti-ethylene compound, to the callus induction medium enhanced pollen callusing frequency in indica cultivars from 10.1 % to 20.6 %. With $AgNO_3$ the frequency of green plant differentiation doubled (Lentini *et al*., 1995). It is also known to promote pollen embryo production in anther cultures of Brussels sprouts (Biddington *et al*., 1988).

Microspore Culture

There are many problems associated with raising androgenic haploids by anther culture. The pollen grains within an anther being genetically heterogeneous, the plants arising from an anther would constitute a heterogeneous population. In cereals, where androgenesis occurs *via* callusing, mixing of calli of different pollen origin within an anther lobe enhances the chances of chimeric plant formation. This limitation can be overcome by isolated microspore culture, which offers many other advantages: (a) it provides an efficient system of isolated, haploid, single cells for biochemical and molecular analysis of androgenesis/ embryogenesis, (b) enrichment of androgenic microspore population by gradient centrifugation becomes possible, (c) isolated microspores can be genetically manipulated before culture to obtain solid mutants or transformants, and selection of new genotypes can be made at an early stage. Unlike the earlier belief, isolated microspore culture is less tedious and time consuming than anther culture.

Considering these advantages, during the last decade considerable progress has been made in isolated microspore culture of cereals (Jahne and Lorz 1995, Raina 1997). Plant regeneration from microspore culture has been achieved in barley (Wei *et al*. 1986, Hunter 1987), maize (Coumans *et al.* 1989; Pescitelli *et al*., 1989), wheat (Mejza *et al*. 1993) and rice (Raina and Irfan, 1998).

Most of the work on rice microspore culture involved an anther pre-culture step (Jia *et al*. 1987; Cho and Zapata 1988,1990; Xie *et al*. 1995). Datta *et al.* (1990) following the float culture method to raise microspore cultures of indica rice cultivars (Basmati-370, Chinsurah Boro II, Upland-13, and a local West Bengal cultivar). Anthers from florets exposed to 4 ^{o}C/10 ^{o}C for 10-18 days were cultured in liquid medium. Within 3-4 days the anthers burst and released the microspores in the medium. The released microspores and those within the anthers were collected and plated in fresh liquid medium. A total of 1300 anthers of the indica cultivars yielded 42 green and 13 albino plants. Against this the Japonica cultivars (Nipponbare, Yamabiko, Yamahoushi) yielded 140 green and 63 albino plants from 3380 anthers cultured.

Recently, Raina and Irfan (1998) reported high frequency androgenesis and plant regeneration in *ab initio* microspore cultures of two indica cultivars (IR43, IR54) and one japonica cultivar (T-309). They observed that co-cultivation of microspores with young ovaries of rice (10 ovaries/ml of medium) significantly increased the androgenic response. However, pretreatment of anthers in 0.4 M mannitol solution for 4 days at 25/30 ^{o}C was essential to induce androgenesis. Another critical factor for sporophytic division in *ab initio* cultured microspores was substitution of sucrose by 9 % maltose. The japonica cv T-309 showed up to 70 % division of the microspores. The response of indica cultivars was comparatively poor but some green plants were obtained for all the cultivars (T-309 9 %; IR43 7 % and IR52 2 %). Some of the indica microspore calli turned brown soon after transfer to semi-solid medium for regeneration. Occasionally, browning was also observed in liquid medium. This problem in barley was overcome by adding Ficoll, a high molecular weight polymer, which keeps the anthers and microcalli afloat on the medium.

Ogawa *et al*. (1992,1994, 1995) achieved androgenic plant formation in *ab initio* microspore cultures of the indica cv IR24 without a nurse tissue. The critical factor was cold pre-treatment of the panicles at 10^{o}C for 28 days before isolation of the micropsores (Ogawa *et al*., 1995). In this study the microspores were purified by filtration and enrichment of androgenic grains by using 0/35% Percoll gradient. They obtained 56 colonies per dish containing 5x10^{4} pollen. Only 6 calli exhibited regeneration and 1 out of 37 plants was green.

Albinism

The occurrence of a large proportion of albinos in the pollen plant population is probably the most frustrating feature of androgenesis in its application to rice breeding. The frequency of albinos may vary from 5 % to 100 %. Indica rice cultivars are more prone to this problem than japonica rice.

Several factors, including pre-treatment, culture medium and the protocol, affect the frequency of albinos. The literature on androgenesis in cereals suggests that albinism can be considerably reduced by shortening the culture period. In most of the cereals androgenesis occurs via pollen callusing and the callus needs to be transferred from the induction medium to regeneration medium for obtaining plants. In rice cv IR43, the pollen calli left on the induction medium longer than 30 d after release from the anthers lost their regeneration potential (Pande and Bhojwani, unpublished). Therefore, we routinely transferred the microcalli to regeneration medium 10 days after their release from the anther. A more effective approach to reduce the culture period is to alter the mode of androgenesis from pollen callusing to direct pollen embryogenesis. In barley, by reducing the culture period to 6 weeks by osmotic shock pretreatment and low density microspore plating, Hoekstra *et al*. (1993) could change the proportion of green to albino plants from 1:1 to 34:1.

Generally, liquid medium is used for anther/microspore culture of barley. Consequently, the anthers and large pollen calli released by them sink in the culture medium, and the tissues are exposed to anaerobic conditions. This results in the production of lactate and alcohol dehydrogenase in the tissues which damages the internal structure and DNA of the plastids, leading to albino plant regeneration (Kao *et al*., 1991). Proper aeration of the tissue by addition of Ficoll (40 %) to the induction medium, supplementing the regeneration medium with Ficoll and high sucrose (4.5 %) and gelling the embryo maturation and germination medium with starch and agar, Kao *et al* induced direct embryogenesis in barley, which cut down the period from anther culture to plant regeneration to 6 weeks. It raised the proportion of green plants from 0 % to 50 %. The authors have remarked that the calli should be transferred from induction medium to regeneration medium within 70 days of culture. High Ficoll / high sucrose kept the anthers and calli afloat on the medium and delayed anther dehiscence, maintaining sugar starvation-like condition around the microspores. The plants regenerated through this protocol showed very little or no genetic variation in the field or with 100 RFLP markers.

The stage of pollen at the time of culture may also contribute towards albinism. In rice the cytoplasm of microspores at the tetrad stage is rich in plastids. With further development simplification of the cytoplasm occurs and the granular matrix of the plastids is replaced by starch grains, which has been positively correlated with deletion of pDNA (Kawata *et al*., 1995). Consequently, calli from anthers cultured at the early uninucleate stage of pollen produce only green plants, and the frequency of albinism increased with advancing stage of the pollen (Pandey 1997). Kawata *et al*. (1995) are of the opinion that more than the process of androgenesis, the culture period is responsible for pDNA deletions. The plants regenerated from one-month-old pollen or seed callus did not show pDNA deletion but those from 11-year-old callus showed substantial pDNA deletion. This theory is further supported by the fact that with the passage of time the calli initially producing only green plants may start producing albinos but it never happens the other way round.

Another observation deserving comment in this context is the *in vitro* selection of microspores. The phenomenon of pollen dimorphism has been observed in several species,

including rice. For example, in IR43 15 % of the grains in an anther are large and thin-walled. These grains are normally incapable of forming viable pollen, which may be due to minor genetic abnormalities. However, these so called androgenic grains are most capable of forming pollen plants in cultures. Albinism may be one of the expressions of this genetic deficiency.

Concluding Remarks

Although considerable progress has been made to improve the *in vitro* androgenic response and the feasibility of *ab initio* microspore culture of indica rice cultivars has been demonstrated, the routine application of this technique to rice breeding is still fraught with many problems. Dihaploid breeding has resulted in the production of several improved cultivars and breeding lines of rice but the success is largely restricted to japonica cultivars. Efforts need to be made to reduce the problem of albinism by manipulating the various *in vivo* and *in vitro* factors and achieving direct pollen embryogenesis to reduce the culture period. In many treatments rice pollen produce rounded, smooth and shining embryo-like structures (ELS) which eventually fuse with adjacent ELS' to form macrocalli. It has been possible to change the 'pollen callusing' pathway to a 'pollen embryogenesis' pathway by manipulating the culture conditions (Kao *et al*., 1991). Gynogenesis is an alternative to raise green haploid plants of rice but the number of haploid plants produced by this technique is a limiting factor.

Figure 2: Androgenesis in anther cultures of IR43. The androgenic pollen form shining globular embryo-like structures (**A**), which later fuse to form microcalli (**B**) rather than embryos.

References

Agarwal, P.K. and S.S. Bhojwani (1993): Enhanced microspore embryogenesis and plant regeneration in anther cultures of *Brassica juncea* cv. PR-45. Euphytica 70, 191-196.

Aruga, K., T. Nakajima and K. Yamamoto (1985): Embryogenic induction of pollen grains of *Nicotiana tabacum* L. Japan J. Breed. 35, 50-58.

Bhojwani, S.S., W.Y. Soh and H. Pande (1999): Morphogenesis in haploid cell cultures. In: W.Y. Soh and S.S. Bhojwani (eds.) Morphogenesis in Plant Tissue Cultures. Kluwer, Dordrecht.

Bhojwani, S.S. and M.K. Razdan (1996): Plant Tissue Culture: Theory and Practice, A Revised Edition. Elsevier, Amsterdam.

Biddington, N.L., R.S. Sutherland and H.T. Robinson (1988): Silver nitrate increases embryo production in anther culture of Brussels sprouts. Ann. Bot. 62, 181-185.

Cho, M.S. and F.J. Zapata (1988): Callus formation and plant regeneration in isolated pollen culture of rice (*Oryza sativa* L. cv. Taipei 309). Plant Sci. 58, 239-244.

Chu, C.C., C.C. Wang, C.S. Sun, C. Hsu, K.C. Yin, C.Y. Chu and F.Y. Bi (1975): Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. 18, 659-668.

Chu, Q., Z. Zhang and Y. Gao (1985): Cytogenetical analysis on aneuploids obtained from pollenclones of rice (*Oryza sativa* L.). Theor. Appl. Genet. 71, 506-512.

Chung, G.S. (1987): Application of anther culture technique for rice (*Oryza sativa* L.) improvement. Res. Rep. Yeongnam Crop Exp. Sta. (RDA), Milyang. Korea. 36-55.

Coumans, M.P., S. Sohota and E.B. Swanson (1989): Plant development from isolated microspores of *Zea mays* L. Plant Cell Rep. 7, 618-621.

Datta, S.K., K. Datta and I. Potrykus (1990): Embryogenesis and plant regeneration from microspores of both 'indica' and 'japonica' rice (*Oryza sativa*). Plant Sci. 67, 83-88.

Datta, S.K. (2001): Androgenesis in cereals. In: Current Trends in Embryology of Angiosperms (S.S. Bhojwani and W.Y. Soh, eds.). Kluwer Academic Publishers, Dordrecht, pp. 471-488.

Finnie, S.J., B.P. Forster, K.J. Chalmers, A.F. Dyer, R. Wough and W. Powell (1991): Genetic stability of microspore-derived doubled haploids of barley: A cytological, bio-chemical and molecular study. Genome 34, 923-928.

Guha-Mukherjee, S. (1973): Genotypic differences in the *in vitro* formation of embryoids from rice pollen. J. Exp. Bot. 24, 139-144.

Gupta, H.S. and D.N. Borthakur (1987): Improved rate of callus induction from rice anther culture following microscopic staging of microspores in iron alum-haemotoxylin. Theor. Appl. Genet. 74, 95-99.

Hoekstra, S., M.H. van Zijderveld, J.D. Louwerse, F. Heidekamp and F. van der Mark (1992): Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Sci. 86, 89-96.

Hoekstra, S., M.H. van Zijderveld, F. Heidekamp and F. van der Mark (1993): Microspore culture of *Hordeum vulgare*: L. the influence of density and osmolality. Plant Cell Rep. 12, 661-665.

Hu, H. (1985): Use of haploids for crop improvement in China. Genet. Manipulation Crops Newslett. 1, 11-23.

Hunter, C.P. (1987): Plant generation method. Australian Patent Application No. AU-A-7271. Shell Int. Res.

Jahne, A. & Lorz, H. 1995. Cereal microspore culture. Plant Sci. 109: 1-12.

Kao, K.N., M. Saleem, S. Abrams, M. Pedras, D. Horn and C. Mallard (1991): Culture conditions for induction of green plants from barley microspores by anther culture methods. Plant Cell Rep. 9, 595-601.

Kawata, M., A. Ohmiya, Y. Shimamoto, K. Oono and F. Takaiwa (1995): Structural changes in the plastid DNA of rice (*Oryza sativa* L.) during tissue culture. Theor. Appl. Genet. 90, 364-371.

Last, D.J. and R.I.S. Brettell (1990): Embryo yield in wheat anther culture is influenced by the choice of sugar in the culture medium. Plant Cell Rep. 9, 14-16.

Lentini, Z., P. Reyes, C.P. Martinez and W.M. Roca (1995): Androgenesis in highly recalcitrant rice genotypes with maltose and silver nitrate. Plant Sci. 110, 127-138.

Lu, C.S., H.C. Sharma and H.W. Ohm (1991): Wheat anther culture: effect of genotype and environmental conditions. Plant Cell Tiss. Org. Cult. 24, 233-236.

Macdonald, M.V. (1992): Donor plant growth factors affecting anther culture of maize and sweet corn (*Zea mays*). Ann. Bot. 70, 357-363.

Matsushima, T., S. Kikuchi, F. Takaiwa and K. Oono (1988): Regeneration of plants by pollen culture in rice (*Oryza sativa* L.). Plant Tissue Cult. Lett. 5, 78-81.

Mejza, S.J., V. Morgant, D.E. DiBona and J.R. Wong (1993): Plant regeneration from isolated microspores of *Triticum aestivum*. Plant Cell Rep. 12, 149-153.

Miah, M.A.A., E.D. Earle and G.S. Khush (1985): Inheritance of callus formation ability in anther cultures of rice, *Oryza sativa* L. Theor. Appl. Genet. 70, 113-116.

Navarro-Alvarez, W., P.S. Baenzigar, K.M. Eskridge, D.R. Shelton, V.D. Gustafson and M. Hugo (1994): Effect of sugars in wheat anther culture media. Plant Breed. 112, 53-62

Ogawa, T., T. Hagio and Y. Ohkawa (1992): Plant regeneration from isolated pollen grains in indica type rice, Japan J. Breed. 42, 675-679.

Ogawa, T., H. Fukuoka and Y. Okhawa (1994): Induction of cell division of isolated pollen grains by sugar starvation in rice. Breed. Sci. 44, 75-77.

Ogawa, T., H. Fukuwa and Y. Ohkawa (1995): Plant regeneration through direct culture of isolated pollen grains in rice. Breed. Sci. 45, 301-307.

Pande, H. (1997): Androgenesis in anther cultures of an indica cultivar of *Oryza sativa* L. Ph.D. Thesis, University of Delhi.

Pande, H. and S.S. Bhojwani (1999): Promotion of androgenesis in rice anther cultures by substitution of sucrose with maltose and mannitol. Biol. Plant. 42, 125-128.

Pescitelli, S.M., J.C. Mitchell, A.M. Jones, D.R. Pareddy and J.F. Petolino (1989): High frequency androgenesis from isolated microspores of maize. Plant Cell Rep. 7, 673-676.

Petolino, J.F. and S.A. Thompson (1987): Genetic analysis of anther culture response in maize. Theor. Appl. Genet. 74, 157-159.

Powell, W.W., T.B. Thomas and D.M. Thompson (1992): The agronomic performance of anther culture derived palnts of barley produced via pollen embryogenesis. Ann. Appl. Biol. 120, 137-150.

Raina, S.K. (1989): Tissue culture in rice improvement: status and potential. Adv. Agron. 42, 339-397.

Raina, S.K. (1997): Doubled haploid breeding in cereals. Plant Breed. Rev. 15, 141-186.

Raina, S.K. and F.J. Zapata (1997): Enhanced anther culture efficiency of indica rice (*Oryza sativa* L.) through modification of the culture media. Plant Breed. 116, 305-315.

Raina, S.K. and S.T. Irfan (1998): High-frequency embryogenesis and plantlet regeneration from isolated microspores of indica rice. Plant Cell Rep. 17, 957-962.

Reddy, V.S., S. Leelavathi and S.K. Sen (1985): Influence of genotype and culture medium on microspore callus induction and green plant regeneration in anthers of *Oryza sativa*. Physiol. Plant. 63, 309-314.

Roberts-Oehlschlager, S.L. and J.D. Dunwell, J.D. (1990): Barley anther culture: Pretreatment on mannitol stimulates production of microspore-derived embryos. Plant Cell Tiss. Organ Cult. 20, 235-240.

Sun, Z. (1999): Methods to increasing frequency of rice anther culture, In: Regional (RCA) Training Course on Production and Utilization of Doubled Haploid Lines in Rice, Republic of Korea, 18-30 October, 1999.

Wei, Z.M., M. Kyo and H. Harada (1986): Callus formation and plant regeneration through direct culture of isolated pollen of *Hordeum vulgare* cv. Sabarlis. Theor. Appl. Genet. 72, 252-255.

Ziauddin, A., E. Simion and K.J. Kasha (1990): Improved plant regeneration from shed microspore culture in barley (*Hordeum vulgare* L.) cultivar Igri. Plant Cell Rep. 9, 69-72.

Application of Recombinant DNA Technology to Studies on Plant Secondary Metabolism

Rafael Zárate[1] and Michael M. Yeoman[2]

[1]Instituto Universitario de Bio-Orgánica A. González, Universidad de La Laguna, Avda. Astrofísico Francisco Sánchez 2, 38206 La Laguna, Tenerife, Spain.
[2]Institute of Cell and Molecular Biology, Centre for Plant Science, The University of Edinburgh, Mayfield Road, EH9 3JH, Edinburgh, Scotland, UK.

Abstract

This review which is concerned with the application of recombinant DNA technology to studies on plant secondary metabolism, presents the more common plant transformation strategies and shows how these genetic approaches are being used in attempts to manipulate and increase the yield of secondary metabolites, both in cultures and in transformed plants. The different plant transformation strategies reviewed here are: infection with intact Agrobacteria; particle bombardment, vacuum infiltration and floral dip; viral vectors and finally protoplast fusion. The review continues with examples of the application of several of these transformation strategies in the manipulation of secondary metabolism. These are outlined under four subheadings which include developmentally regulated genes, addition of novel genes, down-regulation of specific genes and insertion of regulatory genes. Finally, under concluding remarks, reference is made to the advances achieved in the manipulation of plant secondary metabolism and how these approaches may impact on this new

1.- Introduction

Plants produce an array of natural products, the so-called secondary metabolites, which play a variety of roles such as pollinator attractants (e.g. pigments and scents), and defence molecules against attacks by animals and microorganisms. These substances are also important to man as a source of pharmaceuticals, fragrances, agrochemicals and food additives. However, despite great efforts by the chemical industry to mimic and synthesise specific plant secondary metabolites, little success has been achieved and plants still remain the major source of many vital medicinal compounds (Wink, 1990). As most of these compounds originate from plants, any factor (e.g. climatic, political, etc.) which affects the continued supply of these molecules will endanger world supply. In the late 1970s plant cell culture was seen as an alternative, or additional way of producing these compounds, since it was known that plant cells could be readily cultured and produce useful secondary metabolites (see Alfermann & Peterson, 1995). Nevertheless, the low yields obtained with cultured cells, often inferior to the amounts present in intact plants, provided a major drawback to their commercial exploitation. However, a few projects were successful, for instance the production of shikonin and berberine by cell cultures of Lithospermum erythrorhizon and Coptis japonica respectively (Fujita 1988).

Many strategies have been tried in attempts to increase product yield, including for instance the induction of differentiated cell cultures which are known to have a higher biochemical potential (Yeoman & Yeoman, 1996). Indeed, in some cases higher yields of

metabolic intermediates or end-products were attained using this approach. Indeed, established hairy root cultures transformed following infection with Agrobacterium rhizogenes displayed enhanced production of those secondary metabolites which occur naturally in untransformed roots, resulting in amounts of secondary compounds comparable or even higher than those present in intact roots (e.g. Sharp & Doran, 1990; Zárate, 1999). Over the last 10-15 years, the successful genetic transformation of plants has been reported in about 200 species including agricultural crops, trees, ornamentals, fruits and vegetables. Such genetic modification has improved specific crop traits, such as resistance to pathogens, to herbicides and to various environmental factors including drought and floods (Bajaj, 1999). Following on from this success genetic transformation of medicinal plants has been attempted, primarily to enhance the production of various pharmaceuticals, but also flavours, and pigments. Transgenic cultures and plants, have been reported for about 70 of these species (Bajaj, 1999), and interesting results are already appearing. Some of this research was directed towards the control of metabolism. In 1991 Bailey suggested that metabolic engineering of biosynthetic networks might be achieved by application of recombinant DNA methods, but also suggested how complex cellular responses to genetic perturbation could complicate the predictive metabolic design.

In this short review attention is focussed on the different plant transformation methods which have been used successfully and is followed by a series of examples of how these approaches have worked and how they may develop in the future.

2.- Plant transformation strategies

The recent advances and developments in plant genetics and recombinant DNA technology have helped to improve and boost research into secondary metabolite biosynthesis. A major line of research has been to identify enzymes of a metabolic pathway and then manipulate these enzymes to provide better control of that pathway. In this section the most common plant transformation strategies are presented, these include *Agrobacterium* mediated transformation and direct gene transfer. In addition, the applications, advantages and drawbacks of the various methods are also considered.

2a.- Infection with intact Agrobacteria

The gram-negative soil bacteria *Agrobacterium tumefaciens* and the related species *A. rhizogenes* are causal agents of the plant diseases crown gall tumour and hairy root respectively. These species, which belong to the Rhizobiaceae, are natural metabolic engineers able to transform or modify, mainly dicotyledonous plants (Tepfter, 1990), although there are reports on the infection of monocotyledonous plants (Hiei et al., 1994; Ishida et al. 1996), and *A. tumefaciens* has been reported to transform other bacteria and yeast (Bundock et al. 1995).

Agrobacterium rhizogenes has been used regularly for gene transfer in many dicotyledonous plants (Tepfer, 1990). Plant infection with this bacterium induces the formation of proliferative multibranched adventitious roots at the site of infection, the so-called “hairy roots” (Chilton et al., 1982). This infection is followed by the transfer of a portion of DNA i.e. T-DNA, known as the root inducing plasmid (Ri-plasmid), to the plant cell chromosomal DNA. In contrast the tumour inducing plasmid (Ti-plasmid) is present in *A. tumefaciens*.

The Ri-plasmid shares large functional homologies with the Ti-plasmid and appears to have evolved from a common ancestor (Sinkar et al. 1987). The Ri-plasmid consists of a T-DNA, a border sequence and a virulence area. The T-DNA contains the *rol* (root loci) genes

A, B, C, D, which confer the capacity to differentiate into roots on transformed cells. Besides, different opine synthase genes are also present which activate the synthesis of different classes of opines, as well as their catabolism. Opines are unique natural substances, pseudoaminoacids such as octopine, nopaline and agrocinopine, which serve as a nutrient source of carbon and nitrogen, and as specific growth substances for the pathogenic bacteria. For further details the readers are referred to two elegant papers dealing with the different opines and their biochemistry (Petit et al., 1983; Dessaux et al., 1993). The virulence area containing different silent *vir* genes does not enter the plant genome but are required for T-DNA transfer. These genes are activated by wound tissue metabolites, such as lignin precursors and acetosyringone (Melchers et al., 1989) which may explain why tissue wounding appears to be a prerequisite for efficient infection. A large body of research has been devoted to the application of plant transformation and genetic modification using *A. rhizogenes*, in order to boost production of those secondary metabolites which are naturally synthesised in the roots of the mother plant. Transformed hairy roots mimic the biochemical machinery present and active in the normal roots, and in many instances transformed hairy roots display higher product yields.

In a similar fashion, *A. tumefaciens* is also able to infect plant material. A transfer T-DNA is inserted and its Ti-plasmid induces genetic and metabolic changes in the plant cell which result in crown gall disease. These alterations to the biosynthesis of plant growth regulators, which are involved in the regulation of cell division, provoke uncontrolled cell growth and tumour formation. As mentioned for *A. rhizogenes*, the Ti-plasmid of *A. tumefaciens* also harbours genes encoding enzymes responsible for the synthesis and catabolism of the novel aminoacid-like compounds *i.e.* opines which provide a nutrient source. The type of opine formed, such as octopine, nopaline, and agrocinopine, is dependent on the class of *Agrobacterium* strain used and often represents an identifying character to distinguish the strain of *Agrobacterium* employed.

Likewise, as described for the case of *A. rhizogenes*, the process of *A. tumefaciens* T-DNA transfer to the plant genome is triggered by the release of wound phenolic compounds, necessary for the activation of the various *vir* genes which facilitate infection. This complex process is elegantly described by Hooykass (2000). How the T-DNA integrates into the plant genome is not fully understood but it is considered to resemble illegitimate recombination. It appears as a random process with multiple copies often being inserted; nevertheless, once integrated, the T-DNA is maintained stably. Contrary to other gene insertion methods, the plant transgenic lines produced via infection with *Agrobacteria* often contain one copy or a low copy number of the T-DNA, although cell lines with multiple T-DNA copies at one of more loci in the genome can also be encountered. Besides, at much lower frequencies partial, truncated T-DNA copies may be present, or T-DNAs which are accompanied by fragments of the original binary vector. Following this strategy, integration of the T-DNA occurs at random positions in the genome, although there appears to be a preference to insert into transcriptionally active regions.

The importance and advantage of using the *Agrobacteria* system is that by genetic engineering and DNA recombinant technology it is feasible to remove most of the genes in the T-DNAs of both *A. tumefaciens* and *A. rhizogenes*. Subsequent cloning of the desired foreign gene(s) follows, which can then be co-transformed and integrated into the host genome after infection where it can encode specific enzymes dealing with the formation of wanted metabolites or other goals.

In conclusion *A. tumefaciens* has been used directly to genetically modify many plants, and in most cases this requires the tedious and time consuming process of *in vitro* culture and plant regeneration. In addition, two other major problems are encountered when using *Agrobacteria*, these are susceptibility and hypersensitivity of some plant cells or tissues. The

latter leads to tissue necrosis and cell death, although the use of antioxidants such as polyvinylpolypyrrolidone and dithiothreitol can restore plant viability and inhibit necrosis (Perl et al. 1996).

2b.- Particle bombardment

This is another technique of direct DNA delivery, successfully applied to achieve plant transformation; in particular it is effective with recalcitrant species such as most monocotyledons and some dicotyledons. Moreover, it has also been employed to transform animals, fungi and bacteria (Smith et al. 1992; Toffaletti et al. 1993; Johnston et al. 1990). Particle bombardment, also known as Biolistic (biological and ballistic), was developed and first described by Sanford et al. (1987) and Sanford (1988). The technique uses high velocity particles or microprojectiles coated with DNA to deliver exogenous genetic material into the target cell or tissue, which is then cultured *in vitro* and regenerated to produce mature transformed plants.

The particles, either tungsten or gold, are of small size (0.5-5 μm) but big enough to have the necessary mass to be accelerated and able to penetrate the cell wall carrying the coated DNA on its surface which once integrated into the cell nucleus can be expressed. Gold particles are chemically inert, although rather costly, and present more uniformity. Tungsten particles, although with some phytotoxicity (Russell et al. 1992), and of more variable size, are adequate for most studies. Furthermore, the chosen microprojectile should also have good DNA affinity but, at the same time, be able to release it once it has hit the target. DNA coating of surface sterlized particles can be accomplished by binding the DNA, using for instance the calcium chloride method, (Klein et al. 1988) with the addition of spermidine to protect the DNA. However, recently a report describes the novel use of *Agrobacterium* as coating material for the microprojectiles which are then shot into the target tissue, this procedure has resulted in the stable transformation of strawberry plants (Cordero-Mesa et al. 2000). Once coated the particles are ready for shooting and in some cases macrocarriers are employed to support and accelerate the particles. The macrocarrier is retained by a screen or stopping plate and the particles continue travelling and collide with the target. Most frequently, microparticles are accelerated under partial vacuum, and helium pressure may be employed to produce the necessary blast to propel the coated microprojectiles (Finer et al. 1999). Often a spreading screen or metal mesh of specific pore size is located between the point of blast and the target to ensure spreading of the coated particles to achieve a more even collision with the target.

Particle penetration can be controlled by altering several parameters, these include the size of the particles, the distance between the sample holder and the target, the pressure applied to blast the particles, or the presence of a spreading screen used to disperse the particles before hitting the target. In many instances, these parameters are varied to achieve the best conditions to transform a specific material.

The DNA, delivered utilising this direct gene strategy, can be expressed after reaching the nucleus. Presumably most of the coated particles are either degraded or inactivated and only a few reach the nucleus, where some would become stably integrated and expressed and in other cases be expressed in an unexpected manner. Furthermore, frequently fragments of the plasmid used to harbour the gene(s) of interest are also inserted together with intact plasmids resulting in some cases to single insertions or more often multiple insertions. However, a high copy number of the introduced gene(s), does not result in or imply a higher gene expression.

The expression of the gene(s) is dependent on the nature of the DNA as well as the final physical rearrangement of the foreign DNA in the plant genome, the so-called position

effect. When landing in a transcriptionally active region it may be expressed at a high rate, whereas if it integrates within a non-active area, gene expression may be reduced. This appears to indicate that direct DNA delivery by means of particle bombardment does not show a preference for insertion sites, contrary to *Agrobacterium* mediated transformation where DNA tends to be inserted in transcriptionally active regions (Ingelbrecht et al. 1991).

2c.- Vacuum infiltration and floral dip

The vacuum infiltration DNA delivery technique utilises *Agrobacterium tumefaciens* as the DNA vector carrier, together with plants of *Arabidopsis thaliana* at an early flowering stage. The plants are uprooted, and *A. tumefaciens* applied to the intact plants by vacuum infiltration which is achieved by immersion in an appropriate liquid growth medium containing the *Agrobacterium*. Subsequent to replanting, growth and collection of seeds assessment for transformants in the progeny is made by selection using antibiotics or herbicides (Bechtold et al. 1993). Using this procedure, it has been shown, in some detail, that the ovules of *A. thaliana* appear to be the target for *A. tumefaciens* in transformed plants (Ye et al. 1999).

A clear advantage of this approach is that it avoids the necessity for tissue culture and plant regeneration, although it has to be remembered that this strategy has only been successful with a few species, mainly *A. thaliana* but recently with *Medicago truncata*. Clearly species which develop a large number of flowers together with a higher number of set seeds following infiltration are best. In the case of *A. thaliana*, approximately 1-5% would be transformants, equal to 394 transformed seeds per infiltrated plant (Ye et al. 1999).

A novel modification of the vacuum infiltration procedure and a simple method to transform *A. thaliana* is the floral dip (Clough & Bent, 1998). This technique of floral dipping eliminates the labour-intensive vacuum infiltration process. Plants are collected at an early flowering stage, when most of the flowers are still immature. The attached flower buds are immersed in a solution containing a mixture of *A. tumefaciens* cells, sucrose and a surfactant. The appropriate amounts and ratio of these two latter ingredients appears to be crucial for a successful transformation. Furthermore, repeated dipping in *A. tumefaciens* as well as covering the infected plant for one day after bacterial infection, was shown to increase transformation about twofold (Clough & Bent, 1998). After reports of the success of this technique several research groups have applied *A. tumefaciens* by spraying yielding satisfactory results.

Plant transformation following these two methods produces genetically uniform progeny and obviates the somaclonal variation associated with tissue culture and regeneration. Furthermore, these methodologies are not `high tech´ and do not require costly equipment. Unfortunately, very few plant species apart from *A. thaliana* seem to respond to these methods of transformation (Bent, 2000)

2d.- Viral vectors

Several plant viruses have been used as vectors for the insertion of foreign gene(s) into a variety of plant species. However, the quest for a plant virus to enable an easy conversion has not yet been achieved. The large majority of plant viruses have an RNA genome, and just a few are of single or double stranded DNA. Viruses offer large advantages on transient gene expression, they are highly multiplicative, originate a large number of copies of the transgene as well as its expression; moreover, the viruses can propagate easily and spread rapidly to other plant parts and to other plants.

One of the first plant viruses studied was the cauliflower mosaic virus (CaMV) a double stranded DNA virus that replicates through an RNA intermediate (Gronenborn et al. 1981). Despite being considered an ideal candidate because of its small size (8kb) and being

double stranded (DNA), extra DNA insertions are difficult to achieve and often molecular recombinations rapidly eliminate the inserted transgene. Besides, replication of the virus is complex and requires an RNA step which can introduce errors because the inverse transcriptase does not possess a known proof-reading activity, also the CaMV host range is rather limited. Nevertheless, two RNA viruses have been successfully used as vectors. These are the tobacco mosaic virus (TMV) and cowpea mosaic virus (CPMV) (Wilson & Davies, 1992). Two different approaches have been used to clone and insert foreign gene(s) using plant viruses. One is to replace the coat protein of the virus by a transgene or insert the gene(s) next to the initiation codon of the coat protein which will then express the product of the inserted gene and the coat protein. However, in some instances, it has been observed that the inserted gene(s) was removed by the virus thus returning to its original form. Furthermore, the propagation of the virus and the inserted transgene in the plant depend on the presence of a coat protein (movement protein MP) which is needed to spread the transgene within the plant.

Several drawbacks have been reported by those using plant viruses as vectors. These include: their instability, evidenced by the lost of infectivity when compared with intact viruses, and elimination of the transgene when this is larger than 1kb. Unfortunately, the mechanism(s) of viral elimination is not fully understood and therefore not controllable. Indeed, it seems that the ease of insert elimination might be an evolutionary strategy present in the virus to avoid the accumulation of unwanted or unnecessary genomic material which does not provide any advantage.

2e.- Protoplast Fusion

Protoplasts are plant cells in which the cell wall has been removed. Therefore protoplasts can behave like animal cells which have no cell wall barrier. Plant regeneration from single protoplasts is possible due to the totipotency of plant cells, hence plant regeneration from a single cell.

Removal of the cell wall is achieved by treating the plant material (leaves, tissue cultures, suspended cells, etc.) with a cocktail of enzymes including pectinases, cellulases, and/or hemicellulases (Warren, 1991) in an appropriate incubation medium of the right osmolality. After removal of the cell wall, the protoplasts must be kept immersed in a solution of the appropriate concentration to prevent bursting of protoplasts. Also the protoplasts must be incubated in a culture medium of the correct osmolality until wall formation occurs.

Different approaches exist for the insertion of transgenes into protoplasts, either by fusion of different protoplasts or DNA insertion into the protoplast through the plasmamembrane (Lindsey, 1992). These include: (a) chemical techniques, (b) electrical techniques or (c) microinjection. (a) *Chemical Techniques*: several methods have been described, polyethylene glycol (PEG), Ca^{++}-DNA co-precipitation and liposomes. PEG is the most widely used, employing solutions of 10-15% PEG, with high calcium content and high pH. After mixing isolated DNA and protoplasts, followed by different washes, DNA protoplast fusion takes place. Here PEG alters the plasmamembrane properties causing reversible permeabilization enabling exogenous macromolecules to enter the cell cytoplasm. Ca^{++}-DNA co-precipitation: formation of a co-precipitate of plasmid DNA and calcium phosphate is required. On contact with protoplasts, the co-precipitate trespasses the cell membrane in the presence of high calcium concentration and high pH. Liposomes, these are negatively-charged spheres of lipids, are also employed for DNA transfer. DNA is first encapsulated into the liposomes and these are fused with protoplasts employing PEG as a fusogen. (b) *Electrical Techniques*: electrical pulses are applied to the DNA protoplast

mixture provoking an increase in the protoplast permeability to DNA. It is much simpler than the chemical method giving attractive results. However, the electrical pulses must be carefully controlled as cell death can occur above a certain field threshold. These pulses create the transient formation of micropores in the lipid bilayer which last for a few minutes, allowing time for DNA uptake. (c) Microinjection: is an old technique originally designed to transform animal cells, later it gained importance and interest in transforming plant cells. However, in plant cells the existence of a tough cell wall, a natural rigid barrier, as well as the presence of vacuoles which can produce cell death after breakage due to the release of hydrolases and toxic metabolites, and in some instances where vacuoles surround the nucleus make microinjection impossible. Therefore, protoplast rather than intact plant cells are more suitable for microinjection, and thus subsequent genetic modification (Songstad et al. 1995). Clearly, this method is rather labour intensive and requires specialised microequipment for the manipulation of host protoplasts and DNA. However, some success in transforming both monocotyledonous and dicotyledonous species has been achieved employing this technique.

3.- Approaches, systems and metabolites

In the previous section a series of plant transformation strategies were outlined. Here, an attempt will be made to show how these approaches have been used or can be used to manipulate secondary metabolism. It is generally accepted that one of the chief problems encountered when cultured plant cells or tissues are used to accumulate secondary metabolites is low yield of product. Lately, and with the rapid advances made in recombinant DNA technology, it is now possible to tailor secondary metabolism to obtain higher yields of the designated product and to shed some light into the control of these complex metabolic processes. Indeed, to this end many enzymes have been purified, characterised and cloned, and many biosynthetic steps recognised and subsequently manipulated.

3a.- Developmentally regulated genes

It is well known that many secondary metabolites are restricted to a specific organ, tissue or cell type, which may be the site of synthesis, or accumulation or both. Therefore, it would appear that some genes are only expressed in specific tissues, for instance, it is known that some metabolic pathways are confined to roots. For example tropane alkaloid biosynthesis is known to be developmentally regulated and occurs in a specific cell type (Nakajima & Hashimoto, 1999; Suzuki et al., 1999). Thus, *Agrobacterium rhizogenes* transformed hairy root cultures have been used to try to increase the yields of these metabolites, which are naturally synthesised and accumulated in intact plant roots. Indeed, for this reason hairy roots have been employed to study production of root metabolites in a range of species. Hairy roots of *Rubia tinctorum* (van der Heijden et al. 1994) produce comparable amounts of anthraquinones to intact roots with nordamnacanthal, which displays antifungal activity, as the major accumulated compound. Similarly, hairy root cultures of the endangered species *Atropa baetica* (Zárate, 1999) display high accumulation of the major tropane alkaloids, atropine (±hyoscyamine) and scopolamine, with atropine levels similar to intact non-transformed roots. Surprisingly, scopolamine levels were 4-fold higher than intact roots, suggesting a much higher H6H activity (hyoscyamine 6ß-hydroxylase) (Hashimoto et al., 1991) the enzyme responsible of the conversion of hyoscyamine into scopolamine (Fig. 1). Furthermore, and in contrast to other reports on hairy roots of *A. belladonna* producing

tropane alkaloids (Falk & Doran, 1996, Sharp & Doran, 1990), hairy roots of *A. baetica* released considerable amounts of these metabolites into the surrounding liquid medium, and this product accumulation did not seem to be growth associated. Currently, research is underway investigating the effect of overexpressing the *h6h* (hyoscyamine 6ß-hydroxylase) gene under the control of a constitutive CaM 35S promoter in a transgenic hairy root culture of *A. baetica* (Zárate unpublished).

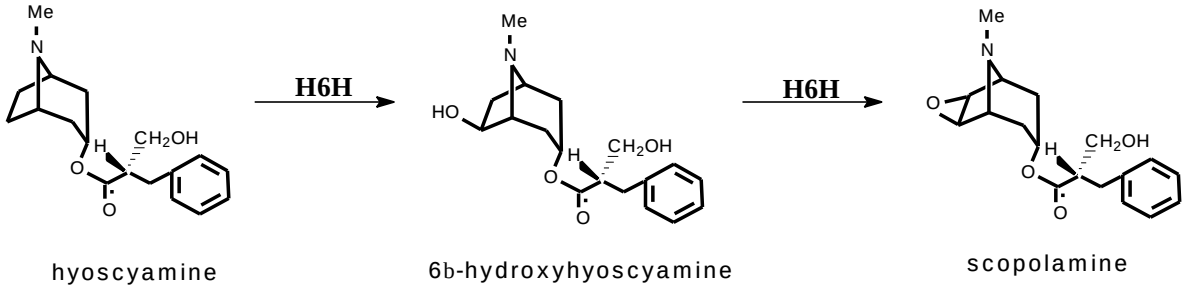


Figure 1: Reactions catalysed by hyoscyamine 6β–hydroxylase, the last steps in the metabolic pathway leading to the synthesis of tropane alkaloids in various Solanaceous species. The enzyme is bifunctional, displaying hydroxylase as well as epoxidase activity.

Progress has also been made in producing genetically modified plants to yield pharmaceutically important metabolites. For instance, transgenic *A. belladonna* plants have been obtained by introducing the *h6h* gene from *Hyoscyamus niger* under the constitutive control of the CaM 35S promoter following the *A. tumefaciens* method (Yun et al., 1992). The regenerated transgenic plants showed a dramatic change in alkaloid composition with scopolamine being the predominant alkaloid present in the aerial parts, and in the branched roots the highly efficient conversion of hyoscyamine into scopolamine was partially enhanced but only small increases were observed. In addition hairy roots of *Hyoscyamus muticus* have been genetically engineered to obtain enhanced amounts of scopolamine over hysocyamine (Jouhikainen et al., 1999). The 35S-*h6h* transgene encoding the H6H enzyme (Fig. 1) was inserted into *H. muticus* via *A. rhizogenes* infection. The authors reported a great variation in tropane alkaloid production among the different transformants with the best line producing over 100 times more scopolamine than the control. However, conversion of hyoscyamine into scopolamine was not total and hyoscyamine remained as the major alkaloid. Furthermore, the expression of H6H was found to be proportional to scopolamine production.

3b.- Addition of novel genes

This is another approach to induce organisms to replicate heterologous cDNA(s) and to express a foreign gene(s) in a host organism. Single step enzymes, as well as part of or all of a complex pathway can be transferred from one organism to another. Recently, an elegant report describes the insertion of a short peptide sequence originally isolated from frog skin, a magainin analog cDNA, to tobacco plants conferring on the plant higher resistance to blue mold disease (Li et al. 2001). A magainin analog peptide Myp30 was found to inhibit spore germination of the oomycete *Peronospora tabacina*, the causal agent of blue mold disease in

tobacco, *in vitro*, and growth of the bacterial pathogen *Erwinia carotovora* subsp *carotovora*, an organism lethal to young tobacco seedlings. Two plant expression constructs were made and introduced into tobacco plants. In one construct (AMY) the peptide was targeted to the cytoplasm, and in another construct (APM) the myp30 sequence was fused with the signal peptide sequence from a tobacco pathogenesis-related protein 1b which targets the Myp30 peptide to an extacellular location. It was found that leaves of tobacco plants expressing myp30 with the peptide directed to an extracellular location (APM) showed a significantly decreased susceptibility to *P. tabacina* infection when compared to control plants and plants with Myp30 peptide targeted to the cytoplasm (AMY). Contrarily, transgenic plants both, those expressing the peptide magainin analog extra or intracellularly, displayed significant resistance to the bacterial pathogen. This Indicates that expression of the gene encoding the magaining type peptide in transgenic plants can increase resistance to the pathogen.

Sekiguchi et al. (1999) have also shown that transformed hairy roots cultures of chilli pepper (*Capsicum frutescens* cv. cayenne) containing the CaM 35S promoter linked to the parsley PAL-2 cDNA displayed an increase in PAL activity at early and late stages of culture. These transformed roots also showed alterations to the metabolism of aromatic compounds such as ferulic acid and lignin-like substances increasing lignification of the induced hairy roots; similarly, the aminoacid content was also altered. Moreover, the increased PAL activity in this culture was associated with slow, abnormal growth of the transformed hairy roots which could be a consequence of increased lignification.

Successful attempts in which the insertion of more than one gene of a known pathway into a host organism have also been reported. For instance, following particle bombardment of tobacco leaves and plant regeneration the expression of two consecutive genes of the terpenoid indole alkaloid pathway of *Catharanthus roseus* ? a well known species able to accumulate the two potent anticancer drugs vincristine and vinblastine? encoding tryptophan decarboxylase (TDC) and strictosidine synthase (STR1) in tobacco plants has been reported (Leech et al. 1998). TDC and STR1 are two adjacent pathway enzymes that together form strictosidine which is an important intermediate of over three thousand indole alkaloids (Fig. 2), many of which possess important pharmaceutical properties. Both *tdc* and *str1* genes are absent in tobacco plants. Analysis of transgenic plants at the RNA and DNA levels demonstrated a range of integration events and steady-state transcript levels for both transgenes besides a 100% co-integration of both transgenes. A comparison of Southern and Northern data suggested that in 26% of the plants both *tdc* and *str1* were silent, 41% demonstrated a preferential silencing of either of the transgenes, with the remaining 33% of the plants expressing both transgenes. The authors did not observe a clear correlation between the number of integration events of a specific transgene and the levels of accumulated transcipt. Although the *tdc* and *str1* transgenes cointegrated into the tobacco genome differential expression of them was recorded. The resulting enzyme activities were indeed very high. TDC originated tryptamine levels of 5-119 µg per mg protein and represented a 5-fold increase as compared with previous published reports. In addition, STR1 activity was 1.1-6.1 µkatal per mg protein, which is 3-22 times higher than at present in *C. roseus*. Therefore, the introduction of these genes by particle bombardment generates diversity at the level of enzyme activity and for *tdc* at the level of product accumulation.

Similarly, the following gene involved in the terpenoid indole alkaloid pathway of *C. roseus sgd* (strictosidine ß–D glucosidase) (Fig. 2) has been introduced via *A. tumefaciens* and expressed in suspended tobacco cells (R. Zárate et al., 2001). The recorded SGD activity in some of the cell lines appeared to be growth associated but in others was steady throughout culture growth, indicating continuous activation of the inserted transgene triggered by the CaM 35S constitutive promoter. Maximal SGD activity of ca. 170 pkat per mg protein was recorded following incubation of a crude protein extract of the transgenic culture.

Furthermore, the molecular data show, as seen in intact *C. roseus* cells, that SGD activity was associated with a protein conformation of 650kDa in size, and this was absent in control or transgenic lines

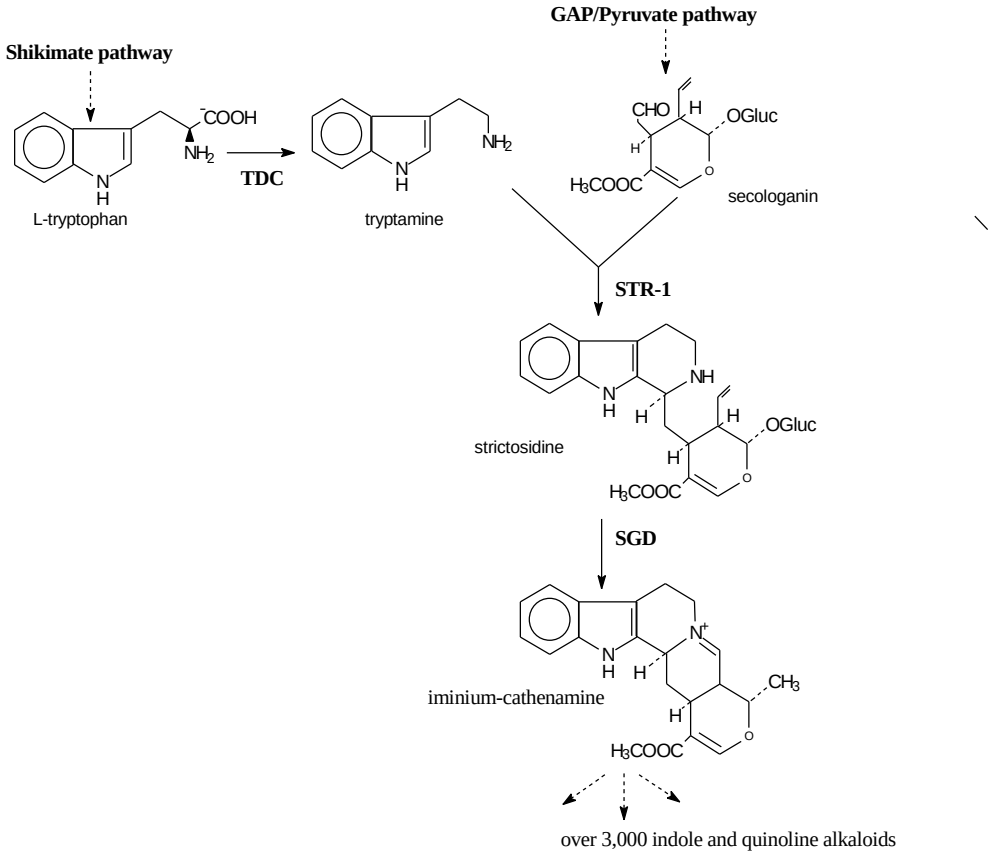


Figure 2: Partial illustration of the biosynthetic pathway of terpenoid indole alkaloids in *Catharanthus roseus* leading to the formation of the intermediate strictosidine, central precursor of over three thousand indole and quinoline alkaloids. (TDC tryptophan decarboxylase, STR-1 strictosidine synthase, SGD strictosidine glucosidase, GAP glyceraldehide-3-phosphate).

which failed to show any SGD activity. Furthermore, genetic transformation of the recalcitrant *C. roseus* plant via particle bombardment has resulted in insertion of the reporter genes (gus, gfp) (Zárate et al., 1999), suggesting that the insertion of the above mentioned terpenoid indole alkaloid genes could be usefully pursued.

There is also an account where a heterologous whole secondary metabolic pathway was expressed in a host plant (Ye et al. 2000) following *A. tumefaciens* mediated transformation. These authors introduced the entire ß-carotene biosynthetic pathway, vitamin A precursor, into rice endosperm in a single transformation effort with three vectors harbouring four transgenes; *psy* plant phytoene synthase, *crt-1* bacterial phytoene desaturase, *lcy* lycopene ß-cyclase, and *tp* transient peptide. In most cases the transformed endosperms were yellow indicating carotenoid formation, and in some lines ß-carotene was the only carotenoid detected. This elegant report illustrates how the nutritional value of a major staple food may be augmented by recombinant DNA technology.

3c.- Down-regulation of specific genes

Many factors can affect a pathway leading to the synthesis and accumulation of a desired end product. These include control by rate-limiting enzymes ? often criticised since most of the enzymes in a pathway are coordinately regulated? , feedback inhibition, caused by the addition or endogenous excess of intermediates, and competition between pathways. Competitive metabolic pathways can be blocked selectively by means of transformation with antisense RNA, RNA transcribed in reverse orientation from ´sense´ or mRNA which binds the messenger sequences and prevents translation. This approach has been employed successfully for instance, caffeine-free coffee plants are being engineered by a research group in Hawaii led by Stiles (see brief report in Newscientist, Coghlan, 1998). These workers identified a master encoding gene (xanthosine-N7-methyl transferase), which governs caffeine production. Via *A. tumefaciens* transformation, the authors introduced an antisense gene construct of this encoding gene which virtually prevents caffeine production in regenerated tissues. Silencing of this gene in all the propagated material appears necessary as the coffee beans did not seem to possess much of the enzyme, although these are the main repositories of caffeine. Enzyme activity in untransformed coffee plants is present chiefly in the leaves, where caffeine synthesis seems to take place which is then transported to the beans. Therefore, the gene would need to be silent during the early developmental stages of the regenerated plants so that there would be no caffeine synthesis and therefore no transport to the beans.

Another interesting gene down-regulation using antisense RNA technology was that contained in an earlier report by van der Krol et al. (1988) who genetically manipulated the flavonoid flower pigments in *Petunia hybrida* by inserting an antisense *chs* (chalcone synthase) gene. This resulted in pigmentation changes in the transgenic plant flowers indicating the potential of this approach.

3d.- Insertion of regulatory genes

A completely different approach to the control of metabolism is by the insertion of regulatory genes or genes inducible by exogenous signals (i.e. elicitors, stress stimulus, etc.) which subsequently activate other gene(s) present in a given pathway, the signal transduction regulatory gene(s). It is widely accepted that biosynthesis of many secondary metabolites in plants can be induced by the stress inducing molecule jasmonate. In *C. roseus* the gene *Orca3*, a jasmonate-responsive APETALA2 (AP-2) domain transcription factor has been isolated (van der Fits & Memelink, 2000). Overexpression of *Orca3* in *C. roseus* cultures resulted in enhanced expression of several biosynthetic genes *tdc*, *str*, *sgd* (Fig. 2), *cpr* (cytochrome P450 reductase), and *dh4* (desacetoxy vindoline 4-hydroxylase) and resulted in enhanced accumulation of certain terpenoid indole alkaloids. However, other genes such as, *g10h* (geraniol 10-hydroxylase) and *dat* (acetyl CoA 4-O--deacetyl vindoline) were not induced, suggesting that these two latter genes were not controlled by *orca3*. These authors also reported that *Orca3* appears as a regulator of primary as well as secondary biosynthetic genes involved in the production of terpenoid indole alkaloids. These findings uncover a control mechanism(s) that may be operative in other cases of stress-responsive secondary metabolite biosynthesis, and opens up the possibility of applying this type of control to alter yields of secondary metabolites (for further reading on this topic see Memelink et al., 2000).

4.- Concluding remarks

In this review, we have attempted to present a range of the more common plant transformation strategies and to show how these various genetic approaches have been employed in attempts to manipulate and increase the yield of secondary metabolites both in cultures and in transformed plants.

Spectacular advances have been reported since an earlier publication (Yeoman & Yeoman, 1996) where the then current approaches for the manipulation of secondary metabolism were reviewed. In addition to the early strategies employed to increase secondary metabolite yield, such as cell line selection, choice of culture system, nutrient regime, level of plant growth regulators, elicitation, precursor feeding, removal of product, and culture conditions (light, pH, temperature, gaseous environment); the full application of recombinant DNA technology and other biotechnological tools are also being applied. This more focussed approach involves the identification of genes encoding metabolic enzymes which will allow full control of a given pathway, together with the manipulation of secondary metabolic pathways, by the insertion of heterologous gene(s) from one organism to another, and attempts to overexpress a single gene to alter the amounts of the end-product of interest.

Despite this surge of research activity the current achievements, although very encouraging, are still limited and many aspects of metabolic control are still unknown and need to be established (for example the involvement of signalling, compartmentalisation, pH, inhibition, activation, enzyme regulation, precursor feeding, feedback effects, etc.). This will take many years to provide a full understanding. Still, the new results are encouraging and stimulating further research which will no doubt provide a framework to give a better understanding of the control of the processes involved in the functioning of a pathway. Hopefully this will lead to a full appreciation of pathway control and eventually enable the manipulation of a chosen pathway to achieve a particular goal.

The application of these ideas and other new approaches, which will presumably emerge, will allow the further manipulation of pathways of different plant species of pharmaceutical or commercial interest and refine the biosynthetic potential of these organisms to make them more productive and profitable, or to alter them to obtain tailored metabolites. We are particularly hopeful that these obstacles will be soon overcome and progress will continue.

Acknowledgements

The authors are grateful to Prof. L. Bender for his invitation to contribute to this special volume. RZ acknowledges financial support from the EU "Qualify of Life and Management of Living Resources" (QLK3-CT-1999-51341).

References

Alfermann AW, Peterson M (1995). Natural product formation by plant cell biotechnology, results and perspectives. Plant Cell Tiss. Org. Culture 43: 199-205.

Bailey JE (1991). Toward a science of metabolic engineering. Science 252: 1668-1674.

Bajaj YPS, Ishimaru K (1999). Genetic transformation of medicinal plants. In: Transgenic Medicinal Plants (ed. Bajaj YPS), pp. 1-29. Biotechnology in Agriculture and Forestry 45, Springer, New Delhi.

Bechtold N, Ellis J, Pelletier G (1993). In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. C.R. Acad. Sci. Paris, Life Sciences 316: 1194-1199.

Beijersbergen A, Den Dulk-Ras A, Schilperoort RA, et al. (1992). Conjugative transfer by the virulence system of Agrobacterium tumefaciens. Science 256: 1324-1327.

Bent AF (2000). Arabidopsis in planta transformation: uses, mechanisms, and prospects for transformation of other species. Plant Physiol. 124: 1540-1547.

Bundock P, Den Dulk-Ras A, Beijersbergen A et al. (1995). Trans-kingdom T-DNA transfer from Agrobacterium tumefaciens to Saccharomyces cerevisiae. EMBO J. 14: 3206-3214.

Chilton MD, Tepfer D, Petit A, David C, Casse-Delbart F, Tempé J (1982). Agrobacterium rhizogenes inserts T-DNA into the genome of the host plant root cells. Nature 295: 432-434.

Coghlan A (1998). Pure brew. Newscientist 157: 11.

Cordero-Mesa M, Jimenez-Bermudez S, Pliego-Alfaro F, Quesada MA, Mercado JA (2000). Agrobacterium cells as microprojectile coating: a novel approach to enhance stable transformation rates in strawberry. Aust. J. Plant Physiol. 27: 1093-1100.

Dessaux Y, Petit A, Tempé J (1993). Chemistry and biochemistry of opines, chemical mediators on parasitism. Phytochemistry 34: 31-38.

Falk LR, Doran PM (1996). Influence of inoculum morphology on growth of Atropa belladonna hairy roots and production of tropane alkaloids. Biotechnol. Letters 18: 1099-1104.

Finer JJ, Finer KR, Ponappa T (1999). Particle bombardment mediated transformation. (eds. Hammond J, McGarvey P, Yusibov V) In: Plant biotechnology. New products and applications. Pp. 59-80. Springer.

Fujita Y (1988). Industrial production of shikonin and berberine. In: Application of Plant Cell and Tissue Culture (eds. Bock G, Marsh J), pp. 228-235. John Wiley & Sons, Chichester, UK: CIBA Foundation Symposium 137.

Gronenborn B, Gardner RC, Schaefer S, Shepherd RJ (1981). Propagation of foreign DNA in plants using cauliflower mosaic virus as vector. Nature 294: 773-776.

Hashimoto T, Hayashi A, Amano Y, Kohno J, Iwanari H, Usuda S, Yamada Y (1991). Hyoscyamine 6ß-hydroxylase, an enzyme involved in tropane alkaloid biosynthesis, is localized at the pericycle of the root. J. Biol. Chem. 266: 4648-4653.

Hiei Y, Ohte S, Komari T, Kumashiro T (1994). Efficient transformation of rice mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J. 6: 271-282.

Hooykaas PJJ (2000). Agrobacterium, a natural metabolic engineer. In: Metabolic Engineering of Plant Secondary Metabolism, Verpoorte R & Alfermann AW (eds.), pp. 51-67. Kluwer Academic Publishers. Dordrecht, The Netherlands.

Ingelbrencht I, Breyne P, Vancompernelle K, Jacobs A, van Montagu M, Depicker A (1991). Transcriptional interference in transgenic plants. Gene 109: 239-242.

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996). High frequency transformation of maize (Zea mays) mediated by Agrobacterium tumefaciens. Nature Biotechnol. 14:745-750.

Johnston SA, Riedy M, DeVit MJ, Sanford JC, McElligot S, Sanders William R (1991). Biolistic transformation of animal tissue. In vitro Cell Dev. Biol. 27P: 11-14.

Jouhikainen K, Lindgren L, Jokelainen T, Hiltunen R, Teeri TH, Oksman-Caldentey K-M (1999). Enhancement of scopolamine production in Hyoscyamus muticus L. hairy root cultures by genetic engineering. Planta 208: 545-551.

Klein TM, Gradziel T, Fromm MF, Sanford JC (1988). Factors influencing gene delivery into Zea mays cells by high velocity microprojectiles. Bio-Technol. 6: 559-563.

Leech MJ, May K, Hallard D, Verpoorte R, de Luca V, Christou P (1998). Expression of the two consecutive genes of a secondary metabolite pathway in transgenic tobacco: molecular diversity influences levels of expression and product accumulation. Plant Mol. Biol. 38: 765-774.

Li Q, Lawrence CB, Xing H-Y, Babbitt RA, Troy Bass W, Maiti IB, Everett NP (2001). Enhanced disease resistance conferred by expression of an antimicrobial magainin analog in transgenic tobacco. Planta 212: 635-639.

Lindsey K (1992). Genetic manipulation of crop plants. J. Biotechnol. 26: 1-28.

Melchers LS, Regensburg-Tuink AJG, Schilperoort RA, Hooykass, PJJ (1989). Specificity of signal molecules on the activation of Agrobacterium virulence gene expression. Mol. Microbiol. 3: 969-977.

Memelink J, Menke FLH, van der Fits L, Kijne JW (2000). Transcriptional regulators to modify secondary metabolism. In: Metabolic engineering of plant secondary metabolism, Verpoorte R & Alfermann AW (eds.) pp. 11-125. Kluwer Academic Press, Dordrecht, The Netherlands.

Nakajima K, Hashimoto T (1999). Two tropinone reductases, that catalysed opposite stereospecific reductions in tropane alkaloid biosynthesis, are localized in plant roots with different cell-specific patterns. Plant Cell Physiol. 40: 1099-1107.

Perl A, Lotan O, Abu-Abied M, Holland D (1996). Establishment of an Agrobacterium-mediated transformation system for grape (Vitis vinifera L.) the role of antioxidants during grape-Agrobacterium interactions. Nature Biotechnol. 14: 624-628.

Petit A, David C, Dahl GA, Ellis JG, Guyon P, Casse-Delbart F, Tempé J (1983). Further extension of the opine concept: plasmids in Agrobacterium rhizogenes cooperate for opine degradation. Mol. Gen. Genet. 190: 204-214.

Sanford JC (1988). The biolistic process. Trends Biotechnol. 6: 299-302.

Sanford JC, Klein TM, Wolf ED, Allen N (1987). Delivery of substances into cells and tissue using a particle bombardment process. J. Plant Sci. Tech. 5: 27-37.

Sekiguchi S, Yamakawa T, Kodama T, Smith S M, Yeoman MM (1999). Characterization of chilli pepper hairy roots expressing the parsley Pal2 cDNA. Plant Biotechnol. 16: 153-158.

Sharp JM, Doran PM (1990). Characteristics of growth and tropane alkaloid synthesis in Atropa belladonna roots transformed by Agrobacterium rhizogenes. J. Biotechnol. 16: 171-186.

Sinkar VP, White FF, Gordon MP (1987). Molecular biology of Ri-plasmid- A review. J. Biosc. 11: 47-57.

Smith FD, Harpending PR, Sanford JC (1992). Biolistic transformation of procaryotes: factors that affect biolistic transformation of very small cells. J. Gen. Microbiol. 138: 239-248.

Songstad DD, Somers DA, Griesbach RJ (1995). Advances in alternative DNA delivery techniques. Plant Cell Tiss. Organ Cult. 40: 1-15.

Suzuki K-I, Yamada Y, Hashimoto T (1999). Expression of Atropa belladona putrescine N-methyl transferase gene in root pericycle. Plant Cell Physiol. 40: 289-297.

Tepfer D (1990). Genetic transformation using Agrobacterium rhizogenes. Physiol. Plant. 79: 140-146.

Toffaletti DL, Rude TH, Johnston SA, Durack DT, Perfect JR (1993). Gene transfer in Cryptococcus neoformans by use of biolistic delivery of DNA. J. Bacteriol. 175: 1405-1411.

van der Fits L, Memelink J (2000). Orca3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289: 295-297.

van der Heijden R, Verpoorte R, Hoekstra SS, Hoge JHC (1994). Nordamnacanthal, a major anthraquinone from an Agrobacterium rhizogenes induced root culture of Rubia tinctorum. Plant Physiol. Biochem. 32: 399-404.

Warren G (1991). Protoplast isolation and fusion. In: Plant Cell and Tissue Culture (eds. Stafford A, Warren G). Open University Press Biotechnology Series, pp. 48-81. England.

Wilson TMA, Davies JW (1992). Genetic engineering with plant viruses. CRC Press Boca Raton, Florida, USA.

Wink M (1990). Physiology of secondary product formation in plants. In: Secondary Products from Plant Tissue Culture (eds. Charlwood BV, Rhodes MJC), pp. 23-41. Oxford, Clarendon Press.

Ye G-N, Stone D, Pang S-Z, Creely W, Gonzalez K, Hinchee M (1999). Arabidopsis ovule is the target for Agrobacterium in planta vacuum infiltration transformation. The Plant Journal 1999 19: 249-257.

Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000). Engineering the provitamin A (ß-carotene) biosynthetic pathway into (carotenoid free) rice endosperm. Science 287: 303-305.

Yeoman MM, Yeoman CL (1996). Manipulating secondary metabolism in cultured plant cells. New Phytologist, Tansley Review no. 30, 134: 553-569.

Yun D-J, Hashimoto T, Yamada Y (1992). Metabolic engineering of medicinal plants: transgenic Atropa belladonna with an improved alkaloid composition. Proc. Nat. Acad. Sci. USA 89: 11799-11803.

Zárate R, Bonavia M, Geerlings A, van der Heijden R, Verpoorte R. (2001). Expression of strictosidine ß-D-glucosidase cDNA, involved in the monoterpene indole alkaloid pathway, from Catharanthus roseus in a transgenic suspension culture of Nicotiana tabacum. Plant Physiol. Biochem. 39 (in press).

Zárate R, Memelink J, van der Heijden R, Verpoorte R (1999). Genetic transformation via particle bombardment of Catharanthus roseus plants through adventitious organogenesis of buds. Biotechnol. Letters 21: 997-1102.

Zárate, R (1999). Tropane alkaloid production by Agrobacterium rhizogenes transformed hairy root cultures of Atropa baetica Willk. (Solanaceae). Plant Cell Rep. 18: 418-423.

Novel Applications of Plant Tissue Culture and Conventional Breeding Techniques to Space Biology Research

Abraham D. Krikorian

Department of Biochemistry and Cell Biology
State University of New York at Stony Brook
Stony Brook, New York 11794-5215 U.S.A.

e-mail address: adkrikorian@earthlink.net

Abstract

In the minds of many, plant cell and tissue culture may now be viewed as a well-developed technology. It has been all but reduced to a tool to solve practical problems and contribute to plant improvement. While this is so in some instances, it is definitely not so in trying to utilize and integrate plant cell and tissue culture into the projected needs of Space biology programs. This is not merely because of the challenges posed by the unusual physical environment of Space such as microgravity, or the constraints posed by Spaceflight protocols but is due to our inadequate knowledge of the factors that control plant growth and development, including systems as they grow in vitro. The premature (and in many cases unrealistically optimistic) initial assessments of what is really known about what happens in aseptic culture belies the many fundamental developmental and physiological questions that still need to be answered before plant "tissue culture technology" can be meaningfully integrated into sophisticated programs and exploited. Nevertheless, opportunities exist to use existing methods. More challenging will be to see novel ways in which to integrate tissue culture strategies into Space biology activities. In the solving of outstanding problems and in the developing of novel approaches, it is anticipated that this knowledge will enable a fuller capability for tissue culture management and utilization schemes here on Earth. Recognition of the still-unanswered basic science questions needing imaginative resolution should in fact stimulate viewing the whole problem in a new and more realistic light. Perhaps "conventional" or "traditional" tissue and cell culture is "dead" (or ought to be dead) but this is a perfect time for the birth of a new era in the use of in vitro systems to study growth and development. This era should, in part, be characterized by a rigorous establishment of what happens when specific media components and environmental parameters are systematically varied. This is literally an area that can enable one with modest resources to "kill two birds with one stone", i.e. basic and applied.

I. Introduction

Retirements inevitably bring to mind new evaluations and in Europe especially, where retirement from Academic life is still somewhat earlier than in the USA, it is especially commonplace that new opportunities often surface for experienced scientists. I myself retired very recently so it is natural that I should reflect on my own career even as I write this paper for a volume being published in honor of Professor Karl-Hermann Neumann, a Senior Scientist whom I have known since our Graduate School days. Indeed, some valuable lessons for others still ‘in full- time service’ may perhaps be drawn from my own attempts over many years to "re-tread" one small

subsection of an area that frequently is seen by non-specialists merely as an “enabling technology" - ready for full application. Professor Neumann will hopefully put his own "old wine into new bottles" after his retirement and in so doing, give many others the benefit of his extensive experience in plant cell and tissue culture. After all, all "retirees"-- real or theoretical -- will be sure to ask and assess for themselves “what did my hard work over many years ‘really’ mean?"

When I was a young and naive scientist, the futuristic aspects of Space plant biology seemed to offer an ideal opportunity which afforded me a chance to participate in the development of tissue culture technologies intended for a novel, even glamorous, environment. An added attraction was that even as one worked in the context of ‘Space and Gravitational Biology’, one simultaneously contributed to the development of a more mature outlook on what various nascent biotechnologies could offer for the benefit of mankind here on Earth. This aspiration, indeed it could be viewed as a need, encouraged me to test, some would say even stretch, the basic science aspects of plant cell and tissue culture to the full extent of my capabilities. The need to ‘put things together’ and make bits and pieces of tissue culture methodology work as a co-ordinated whole was very attractive for it allowed great scope and encouraged vision even as it gave intellectual pleasure and no small degree of satisfaction (cf. Krikorian and Levine, 1991; Krikorian, 1998, 2000).

In this essay an attempt will be made to highlight some of the challenges that I have encountered, and lessons that I have learned. But first, some sort of stage needs to be set for those who are largely uninitiated in this seemingly esoteric area. By the time the reader comes to the end of the paper it will hopefully be apparent that the objectives are not at all esoteric but indeed are part of a unified ‘whole’.

II. Conventional Views of Plant Biology in General and Plant Tissue and Cell Culture in Particular

Many now hold that there is a more-than-adequate grasp of the relevant basic phenomenological aspects of plant cell and tissue culture. This view inevitably leads to the contention that practice of the technology must now perforce be relegated to ‘horticulturists’ and that concerted efforts of ‘basic plant scientists’ must now be directed exclusively to understanding mechanisms–this means full understanding at the molecular level. Nevertheless, some investigators like myself with broad interests have persistently contended that attention must be placed on disclosing, categorizing and ultimately understanding the many outstanding intricacies of all the significant processes involved. From the outset, I understood fully that problems of adapting and applying nominally well-established biotechnologies to the problems of Space Biology would not be trivial and that any imagined line between ‘horticulture’ and ‘basic plant science’ was purely arbitrary and self-serving. Only with an understanding much fuller than was then available would make it possible to utilize technologies fully and in a sustainable fashion. Since the projected uses and concepts were very rudimentary indeed, it was expected that this would take time and would be an evolving technology. Work done by the Soviets was considered rather ‘secret’ and details were often lacking so one had little reliable data base to draw upon (cf. Halstead and Dutcher, 1987 for a review that the Russians themselves said was a better review of what had been done in Space using plants than was readily available to them in the Russian language!). It

may come as a surprise to some that all the essentials of applied “tissue culture”were in place and being practiced in the 1930s! (see Krikorian, 1997; Arditti and Krikorian, 1996).

Nowadays and regrettably, funds for basic, non-applied research are increasingly becoming limited to those studies restricting themselves to molecular biological approaches. Plants like Arabidopsis thaliana (Cruciferae), long appreciated as useful in the study of genetics because of the relatively short life cycle (Meyerowitz, 1994) are even perceived by an astonishing number of scientists as holding ‘the’ key to understanding all important mechanisms in virtually all higher plants. This is supposedly so because its genome nominally encompasses some 90% plus of what is thought to be needed in both basic and practical aspects such as improved agriculture through genetic engineering (Wilson, 2000). But the fact remains that Arabidopsis is a simple plant and accordingly cannot serve convincingly as a model for more anciently evolved and more complicated plant groups.

Let it be clear that I do not dispute that progress on all fronts is necessary and that the use of molecular methods has already contributed greatly to our understanding (cf. e.g. Raghavan, 2000). But ‘all’ the answers are not available yet - not by far, nor will they be I predict in the foreseeable future. I am, however, realistic enough to maintain that there is a need for ever-more imaginative and innovative schemes and projects and initiatives if funds are to be made available so that the still much-needed basic physiological and conventional biochemical work can continue – especially as it applies to plant cell and tissue culture.

Stated succinctly, there remains much to be done in plant cell and tissue culture that is outside the immediate scope of applied horticulture on the one hand, and molecular biology and molecular genetics on the other. Molecular approaches are doomed to be sterile if there is inadequate understanding of the ‘biology’ or ‘natural history’ nominally motivating them. Again, one will be quick to ask: “Who will fund this research aimed at taking full advantage of all modern methods?” Indeed, is a place for plant cell and tissue culture in such schemes justified?

Fortunately, in the USA, the National Aeronautics and Space Administration (NASA) has until recently been a source of funding for some plant research that might otherwise would have been relegated to chance funding. Whether the privileged position that I enjoyed vis a vis tissue culture for space research will be sustained in the future is a moot point. As usual, availability of funds is always being challenged by those who argue for a different perspective. In the course of this essay it will emerge that significant findings on cell and tissue culture systems were facilitated through our addressing specific Spaceflight requirements that in retrospect literally ‘forced’ us to solve long-ignored questions. Had these requirements not existed the work may well have taken alternative turns.

III. Biology in Space: Some Basic Questions

The major issues confronting the US Space Life Sciences research program may be reduced to three questions. (1) Is there a fundamental effect of the Space environment on living systems?; (2) How may we best utilize Space to probe questions of more general importance to the broad field of biology?; (3) How may we best develop and use the foundation of knowledge and understanding that will make long-term manned Space habitation possible and free from major

risks? The settling of these intertwined, and somewhat inconsistent viewpoints and issues using a wide range of organisms will become increasingly important to the International Space program in the years ahead. Few experienced plant biologists should doubt that aseptically cultured plants and plant cells and tissues offer opportunities for study, albeit very challenging ones, in these contexts. In fact, some have gone so far as to say that non-aseptic experiments should not be performed in Space and that even entire Spacecraft should be sterilized for long term missions such as explorations of Mars (Greenberg, 2001).

Whether the answer to the first question posed above turns out to be positive, i.e. some fundamental distinguishing feature(s) of growth and development of plants at near-0 G compared with 1 G emerges; or negative, i.e. no such fundamental feature(s) emerge, either result could have major consequences for any contemplated protracted use of plants in Space. This would apply whether the utilization of plants in a context of so-called 'Space Agriculture', or as components of 'Controlled (earlier referred historically to as "closed") Environment Life Support Systems' (acronym CELSS) (Corey and Wheeler, 1992; Nielsen et al., 1996; Gregory et al., 2000) or as test subjects in experiments which aim to use the unique features of Space to study the effects of gravity on plant growth via "G-unloading" (cf. Keefe and Krikorian, 1983; Krikorian and Levine, 1991, Zimmermann et al.,1988 and refs. there cited.).

The broad concepts on which any effort along the lines just summarized is to be based may pardonably be reduced to the following generalities: (1) That there may be some direct effect of micro-gravity, or the lack of it, upon fundamental processes and/or genetic makeup of cells and organisms; and (2) Except for gravitational effects, there is no significant difference between the Space environment and the environment of Earth insofar as plants interact with it.

How cells manage without gravity and how they change in the absence of gravity are basic questions which only prolonged life on a facility such as a Space Station will enable us to answer. We know from the experience acquired so far from investigations carried out on various kinds of Space vehicles, including Platforms and Stations such as the no longer-existing Soviet Space Station 'Mir' [Peace], profound physiological effects can and often do occur (Nechitailo and Mashinsky, 1993; Tripathy et al., 1996). More needs to be known about the basic biochemistry and biophysics both of cells and of whole organisms in conditions of reduced gravity. Various laboratory activities that are routine on Earth, take on special significance and offer problems that need imaginative resolution before even a relatively simple experiment can be reliably executed on a Space Station. For example, scientists will even have to investigate whether adaptive or other changes which have occurred in the environment of Space are retained after return to Earth-normal conditions. Otherwise, one will perforce end up having an isolated and parochial discipline under the aegis of 'Space Plant Biology'. Limitation of research specifically to the micro-G environment would surely endanger any research effort. Some sort of connection with Earth-normal biology is crucial.

Gravitational plant biologists have, of course, given considerable thought to the kinds of changes in response that might result from exposing plants to micro-G in the Space environment. There are a number of situations in which orientation with respect to 1-G are already known to alter the response. Gravitropism of organs, especially roots and shoots is well known, has been

extensively studied and the role of specialized cells, or statocytes is known to be central to this phenomenon. Gravitaxis has been much less extensively studied. Similarly, gravi-morphogenesis, such as the formation of reaction wood, the breaking of buds and its relationship to apical dominance, or the determination of position of organ emergence or even the determination of the type of organ or cell formed have been very inadequately studied (Sack, 1991, 1998; Digby and Firn, 1995).

In addition to these categories of inquiry, one can expect as yet unidentified situations where the 'Earth normal' (1-G) condition is required - that is, micro-G might be expected to eliminate a response, i.e. have a qualitative effect. These are essentially unidentified or unknown at present; indeed virtually all aspects of plant physiology and development are potential candidates. It will not be a trivial matter to disclose these situations and to validate them using rigorous scientific methods (Barlow, 1995).

Last but not least are instances where micro-G would be expected to alter a response, i.e. have a quantitative effect. For example, the extent and nature of lignification might be expected to be different in micro-G because a system undergoing lignification would be less ‘G-loaded’. Also in this category, and minimally understood at present, is the area of investigation which asks the question "What effect does micro-g have on cells that are not specialized for G-sensing?" And, "What effect does micro-G have on developmental, physiological and reproductive processes?" etc. (Krikorian, 1996a and b, 1998).

Skeptics and critics has emphasized that there is no fundamental or constitutive short-term, or acute effect of gravity, or absence of it, upon plants and their cells. Or, stated another way, it is irrelevant to consider the question seriously for manned spacecraft and Space Stations such as the one being presently assembled because there will always exist a certain 'above-threshold G environment' associated with either on-orbit maneuvering or human activity (Keefe and Krikorian, 1983; Krikorian and Levine, 1991). Conversely, in terms of the practical functioning and growth of plants, there is a profound effect of the altered physical environment associated with micro-gravity. This boils down to somewhat semantic arguments of "direct" vs. "indirect" effects of micro-G. On this simplistic argument, the "direct" effect is mediated, only to a minor extent, through such phenomena as the sensing and orientation response. (It is a truism that changes in organisms during the last 500 million years are due largely to the rearrangements of basically similar building blocks-cells-by gene regulation mechanisms and not by the creation of new genes. In other words, basic metabolic patterns were already in place and new kinds of organisms were produced by rearrangements of the same biochemistry in different building blocks. I contend that microgravity/space must have effects on processing of cues of various sorts and the first step is to define what these effects are –Markert and Krikorian, 1993.)

On this line of argument, much more important are the indirect effects of the physical environment. One may ask, for example, what is unique about the low G environment with respect to Space experiments involving fluids? In the first place, although the laws of classical physics such as momentum and mass conservation, energy conservation, Maxwell's equations etc. still apply, the relative importance of the G force to other forces changes. Also, the fact that experiments are being conducted in a non-inertial frame becomes more important and the effects of variation of g become more important. Moreover, in the near-weightless environment of Space (here weightlessness is defined as $10^{-6 \text{ to } -4}$ G), there are no convective currents, no buoyancy, and surface tension dominates. Consequently, because of the dominance of surface tension/molecular attraction, fluids tend to deposit in unexpected or undesirable ways on plant surfaces and growth media, greatly impeding air movement. The exchange of energy and metabolic gases between the plant and its liquid or gaseous environment will be radically different in space because of the absence of gravity-driven convection. Diffusion, convection, active mixing, and asymmetrical distribution of particles all will be different in Space. But the fact is that information on the precise nature of these phenomena is sparse and has been the subject of only limited study and that by engineers in a non-biological context (Benedikt, 1960; Claaseen and Spooner, 1994). There is no way of predicting what the exact nature of the convective environment in micro-G will be. Studies on Earth indicate that absence of G-driven convection will have considerable impact on the normal gas and heat exchange phenomena that are important to "normal" plant physiological function. Even now, there is reasonable, albeit circumstantial, evidence that there may be effects of the lack of gravity-driven convection that have significant impact on plant growth (Musgrave et al., 1997). Altered aeration and gas exchange in the environment of roots may well provide one component of an explanation for observed anomalies in space-grown materials such as cell structure, atypical cytological characteristics such as chromosomes which are ruptured or otherwise altered, and poor mitochondrial development (Nechitailo and Mashinsky, 1993).

Similarly, biological manifestations of these indirect effects may range from far reaching to insignificant depending on the biology of the system, and the culture environment. It logically follows that if equipment for experimentation such as plant growth chambers either for basic science experiments or for practical functions are to be designed properly, it is

important to characterize as rigorously as possible the interaction between micro-G, other physical environmental parameters, and plant responses (cf. e.g. Salisbury, 1991 a and b; Nielsen et al., 1996).

It will come as no surprise that NASA has recognized for some time that a number of issues regarding plant growth in Space need resolution (Table 1). Emphasis, of necessity, has thus far been placed on study of short term effects or adaptations or responses to Space. This should be distinguished from long term adaptations compatible with a true biology of plants in Space.

Table 1: Some Expected Changes in Plant Response as a Result of Exposure to micro-G in the Space Environment

A. Situations in which Orientation with respect to 1 G is already known to alter the response:
gravitropism
  organs, especially roots and shoots, specialized single cells, e.g. statocytes
gravitaxis
gravimorphogenesis
  formation of reaction wood
  bud break/apical dominance
  determination of position of organ emergence, determination of the type of organ or cell formed
epinasty

B. Unidentified situations where 'Earth normal' (1 G) is required--that is, micro-G might be expected to eliminate a response, i.e have a qualitative effect:
Unknown at present, everything is a potential candidate

C. Situations where micro-G would be expected to alter a response, i.e. have a quantitative effect:
Lignification?
Unknown at present, e.g. What effect does micro-G have on cells that are not etc. specialized for G-sensing, or on developmental, physiological and reproductive processes?

Some effort has gone into evaluating directional responses as a result of reduced G, i.e. tropisms (Brown, 1996). Altered tropistic responses are not, however, the most important effects plants will show when grown in the reduced gravity environment of Space. They probably have little direct significance for early or primary development of plants in Space but they still may have indirect effects (Digby and Firn, 1995).

Nevertheless, marked changes in plant appearance due to disturbances in orientation in "above"-ground or "below"-ground organs, in epinasty of roots and leaves, and diminution of nutational movements all have been reported (Dutcher et al., 1994; Nechatailo and Mashinsky, 1993). Since phenotype is of adaptational significance, this will have effects on biomass. It is necessary to study qualitatively and quantitatively the patterns that relate changes in plants to parameters that have significance for growth, development and metabolism. There are many questions of importance to plant growth in Space. Changes in biochemistry, reflected in such effects as replacement of cellulose with hemicellulose and associated anatomical disturbances due to ‘G-unloading’ on support tissues such as lignin may be expected. Modifications in water transport mechanisms and a consequent change in vasculature may also be predicted (Sachs, 1991).

All the above indicates that the way in which things are exposed, ‘grown’ if you will, in the Space environment will impact tremendously the results.

Understandably, the objective of an experiment normally dictates the way in which it should be grown. But I have already emphasized that the database for growing plants in Space is minuscule and the near-term and increasingly urgent requirement is to move step-wise towards developing systems for the reliable growth of plants in Space (Salisbury, 1991a and b; Corey and Wheeler, 1992; Musgrave et al., 1997.)

IV. The Controlled Ecological Life Support Systems (CELSS) Challenge

A controlled environment agriculture is often seen as a critical component of man's ability to achieve a “permanent presence” in Space. This permanent presence will at the outset necessarily be acute--say order of three to six months--but it will increase in duration to chronic, very much longer levels, eventually becoming even multi-generational (Pirie, 1980; Mitchell, 1993). As part of the human support capabilities, there will be a need for advanced life support systems. A CELSS as now envisioned by many in the USA, Europe and Japan as well, will rely on biological means, whereas an "open" one will emphasize a purely chemical approach and ‘resupply’ parameters. Various forms of plant life are central to the whole support effort but plants will also predictably play roles other than as a source of nutrition, and a means of atmospheric control or waste processing (cf. e.g.Wolverton et al., 1983, 1984). This role has been, for lack of a better description or heading, categorized under “Human Factors”. The mere presence of, or access to plants in the ultimately monotonous and relatively confined environment of even large Space facilities, and even the supplementation of a perhaps otherwise highly processed and unimaginative diet with a few fresh greens will undoubtedly have a positive effect on the human psyche. In fact a ‘Salad machine’ has been proposed (Kliss and MacElroy, 1990).

If one views the overall problems in the context of food production, waste processing and their control and management, then the question arises as to what extent can newer plant biotechnologies play a role in enhancing the efficiency of, or managing, these kinds of anticipated activities? The next section addresses these issues.

V. Background to the Use of Cultured Cells and Tissue

Readers will appreciate that activities by tissue culturists over the past twenty-five years have dramatically increased capabilities for problem solving. There now is a massive literature that seeks to provide the interface between innovative breeding and various plant propagation and management practices, and the challenges and opportunities posed by particular species (Lindsey, 1992; Vasil and Thorpe, 1994; Bajaj, 2000). The most obvious of the potential uses of plant tissue culture technology in a CELSS setting involves relatively rapid multiplication of higher plants (Keefe and Krikorian, 1983).

Table 2 provides a list of various in vitro strategies which can lead to multiplication of desired ‘bio-specimens’, whatever the intended use. Whereas the term "tissue culture" or in vitro culture is largely used in a generic sense, and hence does not necessarily connote a precise strategy, the enumeration in Table 2 provides in greater detail a number of the different strategies than one can adopt in an effort aimed at yielding increased numbers of propagules or plantlets. These range from the relatively well-established procedures of (1) fostering various levels of branching or shoot development by releasing (usually hormonally) various corelational controls normally in place in the intact plant body; (2) inducing organized growth or direct organogenesis from excised organ or tissue systems without an intermediate and extensive callus stage; (3) fostering de novo organized growth in the form of shoots and roots from callus systems; to (4), the much less predictable stimulation of the formation of somatic or non-zygotic embryos from so-called morphogenetically competent cells and tissues (cf. Soh and Bhojwani, 1999).

Table 2: Strategies for Multiplication of Higher Plants in vitro.

- Shoots from terminal, axillary or lateral buds
  . shoot apical meristems (no leaf primordia present)
  . shoot tips (leaf primordia or young leaves present)
  . buds
  . nodes
  . shoot buds on roots
- Direct organogenesis
  . adventitious shoot and/or root formation on an organ or tissue explant without an intervening callus
- Indirect organogenesis
  . adventitious shoot and/or root formation on a callus
- Somatic embryogenesis
  . direct formation on a primary explant
. 'direct' formation from embryo-equivalent cells grown in suspension or on semi-solid media
- Direct plantlet formation via an organ of perennation formed in vitro
- Micrografting
- Ovule culture
- Embryo rescue
- Mega-and microspore culture
- Infection with a crown gall plasmid genetically altered to give teratoma-like tumors

Additionally, plantlets can be generated directly from organs of perennation which are sometimes controllably and precociously inducible in vitro. Similarly, micro- grafting performed in vitro can effectively lead to more plants in some cases. Ovule culture can in certain cases rescue or lead to plants that would otherwise be lost, and embryo culture can provide the same capability. Mega-and microspore (pollen) culture can likewise lead to materials that reflect either the genotype of the female or male germline respectively. A much more tenuous method uses infection with a genetically altered plasmid of the crown gall micro-organism (Agrobacterium tumefaciens) that lead to teratoma-like (tumorous) structures from which whole plants can ultimately be recovered (cf. Ream and Gelvin, 1996).

All these strategies can, then, be invoked with varying levels of efficiency and technical finesse towards the end of multiplying plantlets in vitro. No useful purpose is served here in extensively discussing each of these strategies. The works referred to above address the specifics and provide detailed perspectives on the precise range of capabilities. In theory at least, virtually all plant species are amenable to manipulation. Nevertheless, it follows that there are a very large number of parameters that must be taken into consideration if any of these strategies is to be optimized in the Space environment, just as the many environmental parameters must be addressed in depth here on Earth (see Kozai et al., 1992 and Fig.1). Convective mass transfer and liquid atomization are but two examples of major problems that must be overcome in Space (see Prince et al., 1991 and Bayvel and Orzechowski, 1993 respectively.)

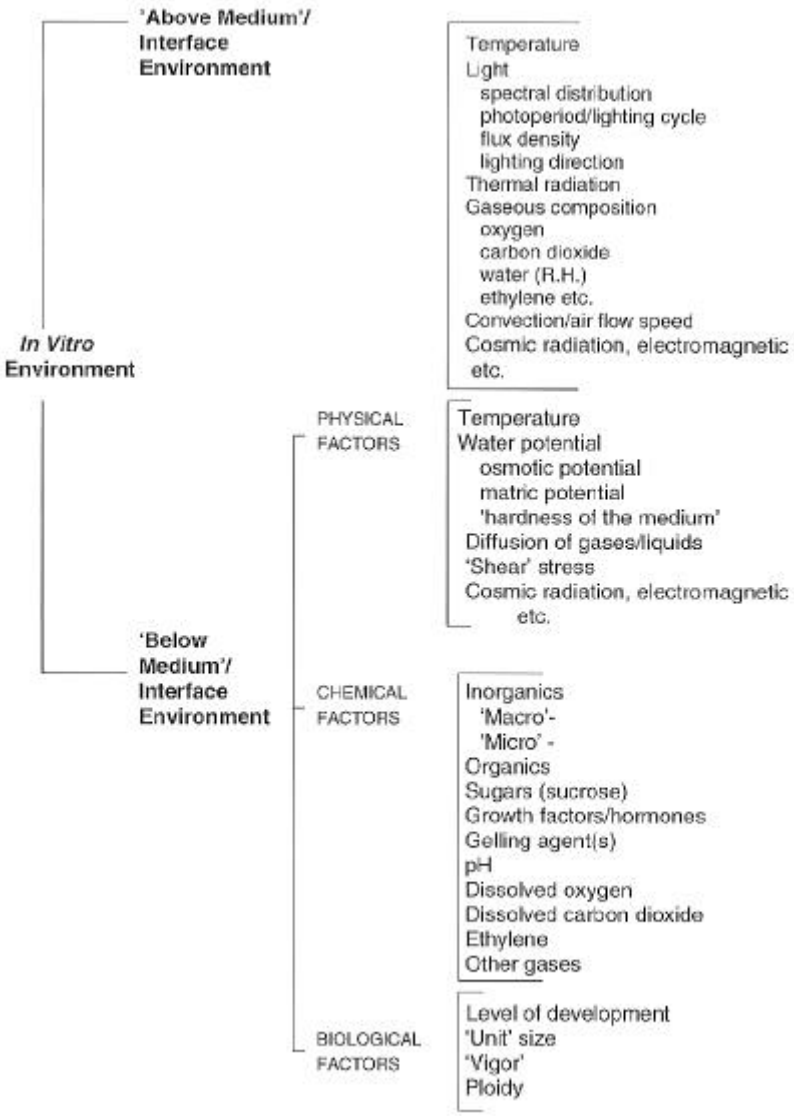


Figure 1: Some macro- and micro-environmental parameters affecting development and growth in vitro (Inspired by Kozai et al., 1992).

VI. Gravitational and Developmental Studies Using Higher Plant Cells in the Context of Modern Biotechnologies

Plant biotechnology is, of course, a broad, complex field, and there is considerable overlap of basic science, technique and technology into areas usually termed "industrial biotechnology" and "chemical engineering". It is moreover, a rapidly moving field. Even so, numerous opportunities still exist for integrating and, indeed, taking advantage of a number of distinctive research perspectives or capabilities which are currently of special interest to various Space Agencies. At the same time, these same research opportunities and approaches, if better integrated and co-ordinated, have high potential for broadening the science and technology base and strengthening interdisciplinary approaches here on Earth.

This is sure to optimize 'science return' and to contribute more effectively to a increasing our basic understanding of a host of questions with both basic and biotechnological importance. Integrating the two areas will require that practitioners from various disciplines work together to identify the critical needs and capabilities of each.

One especially salient example follows:

Bioreactors

The culture of plant cells in bioreactors is increasingly being seen as having substantial potential in the biotechnology industry for the production of high cost biochemicals, enzymes, and other distinctive secondary products etc. On Earth, because of gravity, the content of a bioreactor must be mixed in order to obtain a proper distribution of nutrients, oxygen, temperature and pH environment. This mixing creates a harsh hydrodynamic shear environment detrimental to sensitive plant cells. If not mixed properly, cells tend to congregate, and by zone sedimentation, fall to the bottom of the bioreactor. Furthermore, the requirements for oxygenation creates a foaming in the bioreactor which also tends to perturb and otherwise damage cells. These factors limit the concentration and density of the bioreactor nutrient culture medium. On Earth, it is known that concentration and density of the solution are directly linked to the optimal performance of bioreactors; the higher the density, the more cost effective the bioreactor 'run' (Doran, 1993; Wilson and Hilton, 1995).

In microgravity, zone sedimentation disappears, which should reduce the aggregation of cultured cells. Moreover, only gentle mixing is required to distribute nutrients and oxygen. These factors should permit higher concentrations and densities to be achieved in a low G environment. Additionally, since the cells do not need to maintain the same surface forces that they require in Earth-normal gravity, they can divert more energy sources for growth and differentiation and in theory, at least, the biosynthesis of more product, or even novel products the production of which would be unpredictable (Cogoli and Tschopp, 1982; Cogoli and Gmünder, 1991). Because one can impose variable gravity on these cell systems, one has the means to test the consequences of increasing or decreasing G on secondary product biosynthesis.
Some work has already been carried out on plant cells in a Bioreactor setting in Spaceflight. Results indicate that metabolism, productivity and differentiation characteristics of a variety of cells is altered. This might be due to decreased cell interactions (contacts) when cells are freely suspended (Krikorian, 1996a). Clearly, there are many opportunities to study these responses and these are sure to lead to a better understanding of the mechanisms by which plant cells control production of secondary metabolites and other cell products. With this knowledge, control of enhanced, sustained production of product by plant cells might be possible. See Durzan (2000) for a detailed analysis of metabolic engineering and specific plant biosyntheses in a Space environment.

There is, then, a great need to pursue specific biosyntheses under various controlled microenvironments. The study of mechanisms in the context of bioreactors is rudimentary and only when much more is known will one be able to move forward. Sophistication of available cell culture chambers or bioreactors has steadily been improving and opportunities for increasing our knowledge base is virtually unlimited. Capability to study inter- and intracellular, even subcellular gaseous environments, combined with study of compartmentalization of various key signaling events and sensing components within cells, as well as the targeted manipulation of pools and nutrients with novel agents such as chelators, all provide means to disclose and understand mechanisms.

The use of molecular markers such as monoclonal antibodies to bind to specific sites and the cloning and analysis of cDNA encoding a specific synthesis could provide yet another level of detail in pursuing the nature of basic control mechanisms. Study of inducible control of gene expression in cultured plant cells in a Space environment is certain to disclose new and unexpected findings (Reynolds, 1999).

VII. Gravitational Biology and Mechanisms which Control the Differentiation and Development of Plant Cells, Tissues and Organs in vitro: Relevance to Emerging Plant Biotechnologies

One of the major constraints to progress in genetic engineering of higher plants continues to be the ever-present lack of a complete understanding of the controls which permit cells to express their innate potential to multiply and embark on a pathway of development that approximates that of zygotic cells (Thorpe, 1995; Neumann, 1995; Raghavan 1997; Soh and Bhojwani, 1999). A major objective is to recover plants from such cells via a developmental pathway involving production of somatic embryos for use not only as an end unto itself for basic research, but in a Life Support System, and as means to store germplasm for a multitude of purposes in Space (Keefe and Krikorian, 1983; Krikorian, 1996a). Yet another impetus for this kind of work is that the understanding of what controls totipotency and genetic expression is often fundamental to operations wherein new genetic material may be introduced into plant cells (Soh and Bhojwani, 1999; Raghavan, 1997, 2000).

Significantly, NASA has for some time been concerned with problems of so-called plant “gravimorphogenesis” and a major question has been whether proper polarity can be achieved in single cells in an environment where gravity signals have been "eliminated or erased ". Early on in my Space biology investigations, the seemingly cogent argument was made that totipotent cultured cells offered substantial merit for studying the question whether polarity could be established in the absence of G vector - or more accurately in an environment where the G vector was more or less neutralized, as in a clinostat (Hoshizaki, 1973; Brown, 1996; Krikorian, 1998). The argument went along the following lines. If one, in theory at least, could work towards determining thresholds for any number of parameters, it should be equally interesting to ascertain yet another type of threshold level. To me, a particularly interesting question was "What constitutes ‘the’ or ‘a’ minimal G-responsive unit from the perspective of development? The theory of totipotency has, of course, historically been predicated on the view that individual cells are capable of giving rise to entire plants and it seemed perfectly reasonable to ask the question whether free cells could establish polarity and continue to develop in Space.

The cell that is the classical example of this presumed need to establish polarity, perforce, is the fertilized egg or zygote which can grow and develop into an embryo and from that sprout into a whole plant (Vroemen et al., 1999). Why not, merely then, take seeds and use them as experimental objects to determine development thresholds? One could I argued, indeed, do this but one would be determining whether an embryo could develop or progress from one stage to another, under a measurable G load. Because the embryo is generally very well developed in most seeds, one cannot study very early developmental events using seeds which are separated from the parent plant (the sporophyte). If one wanted to use early stages of development, one had to have the fertilized egg in place (in situ) in the embryo sac of the ovule (which will develop into the seed) or, one had to have lots of isolatable, fertilized egg cells that can grow. The earlier in development the better, since from the earliest stage, all

else grows. To have as complete a picture as possible, one obviously wanted to be able to expose a broad spectrum of stages to the Space and a range of hypo-G environments. (Again the indispensability of access to a centrifuge for use in Space became apparent.) These are not easy points on a curve to determine, however, because what one is essentially being challenged to do is to expose fertilized egg cells removed from the embryo sac of a higher plant and to allow them to develop under controlled conditions into a fully mature embryo - passing through all the classical stages of embryogeny, all the while assessing their performance at specific G levels. Each of the stages would have to be pre-determined to be amenable to mass collection, in the first place, so as to have ample supply for experimentation. Then, assuming one had enough of them at any of several stages of development, one had to be able to grow them on Earth. This assumes one has the skills and understands the requisite nutrient requirements to bring the developing embryos through to their full level of growth. Then one has to be able to "package" them for flight experimentation. All this was a major challenge - and given the state of the technology, it was impossible to do. Hence the argument that totipotent cells would provide a more tractable substitute for zygotes. [It had not been convincingly shown that embryogenic cultures grown in suspensions were already zygote-equivalents, that is they were already determined (Krikorian, 2000). I vacillated on this point of exactly when determination occurred. That was because of several unresolved parameters. Had I known this for certain no Space flight experiments would have been proposed using cultured plant cells! Or, at least the question asked would have been posed in a different context.]

If one adheres to the line of reasoning started above and brings it to its logical conclusion, we can come to the viewpoint that the degree of sensitivity or responsiveness to G varies at different degrees of organization or stages of developmental complexity. Experiments which impose varying G force levels on different degrees of "developed material" should permit us to pinpoint the degree of prior organization, if any, at which salient problems might arise in the Space environment. This should teach us, my argument continued, what are the minimal G forces required for normal plant development (and physiology), and through centrifugation in Space, the maximum G force that be tolerated before a gravitropic response is elicited. By "erasing G signals" one could investigate what happened.

Indeed the very first experiment using embryogenic cells (then simplistically referred to merely as ‘totipotent’ cells) of a higher plant in Space was performed on the Soviet unmanned satellite BioKosmos in 1975. Initially the conclusion was drawn that somatic embryogenesis proceeded unhindered in the weightless environment (Krikorian et al., 1981). Somewhat later, a more sophisticated analysis of data showed that there was in fact a blockage in the normal progression of somatic embryogenesis from ‘free’ cells in vitro beyond the globular stage and that there was a failure to polarize (Krikorian, 1991, 1998). More recent investigations using Dactylis glomerata have confirmed and extended our pioneer observation. That Spaceflight reduces somatic embryogenesis from primary explants as well as from already-embryogenically determined cultured cells derived from embryogenic suspensions is not surprising (Conger et al., 1998). Opportunities to probe in greater depth whether this constitutes a limitation of specific receptors to function in low G or whether it is ‘merely’ a reflection of the technology of the system being tested will be at once apparent (see more on this later).

Additional to work on somatic embryogenesis, and from the perspective of bioreactors and stage-specific biosynthesis, should constitute a unique opportunity to ascertain whether specific syntheses can be sustained in Space in ways not possible on Earth. As of yet, there is no way to non-invasively stop somatic embryo development at a specific stage, and hence the

conventional approach to the study of stage-specific biosynthesis has been to mechanically isolate the stage in question being sought. This is much harder to do than might be apparent. Synchronization is no simple problem.

Use of pH and Reduced Nitrogen to Control Development of Embryogenic Cells

Conceptual approaches related to Spaceflight experiment implementation in the context of seeking an understanding as to whether there are developmentally-related limitations to gravity sensing, i.e. “Is there a morphologically 'minimum' unit required for g sensing?” has led to the appreciation that as-simple-as possible-controls must be sought in embryogenic or developmental models. In the course of this work, an innovative and important mechanism based on pH was discovered in my laboratory which controls transition from preglobular stage embryos to globular and later stage somatic embryos. The mechanism, whatever the basis of it is, is not restricted to a few species for it has been shown to be operative in each of the test systems we studied (cf. Smith and Krikorian, 1990a and b, l991, 1992).

Briefly, we showed that embryogenic cultures of carrot and other plants can be initiated and maintained with continuous multiplication using a hormone-free medium. In the course of this work we also showed that high frequency production or initiation of embryogenic globules from wounded zygotic embryos is dependent upon the use a medium with a pH above 4.5 and NH_4^+ as the sole nitrogen source. However, maintenance with continued multiplication of unorganized, embryogenic cell masses requires that the pH of hormone-free, NH_4^+-containing medium be maintained at or fall to 4 during each culture period. If the medium is buffered at or above pH 4.5, embryogenic globules continue to develop into later embryo stages (Smith and Krikorian 1989, 1990a and b).

In the case of carrot cultures, preglobular stage embryos - that is to say the stage most reminiscent of the zygote - before the proembryo or globular stage embryo forms - can thus be kept "cycling" and increasing in number provided the pH is kept low. If the pH is elevated, the somatic embryos continue their development and proceed through the "normally expected" stages of embryogenesis - globular, heart, torpedo and cotyledonary (or the equivalent in a monocotyledonous system).

The "simple" parameter of pH under these circumstances should not be viewed as a sort of second messenger but merely a case of providing an inappropriate, non-stressful environment for embryogenic progression. For years one has heard and read the dogma that the "best" pH for a cell culture medium is such and such (usually around pH 5.6-5.8 or so). Clearly this now needs to be qualified and a further statement made about what situation one is talking about. If one wants more preglobular stage somatic embryos (or embryogenic cells or cell clusters), then the pH should be kept low (below pH 4.5). (It should again be emphasized that the low pH does not confer embryogenic status or capacity on the cells. The pH works only on cells that are already in the embryogenic mode. It is a modulating agent---an important one--not an inducing one (Smith and Krikorian, 1990a; Krikorian and Smith, 1992) . Another factor, long appreciated as important, involves nitrogen supply--be it ammonium or nitrate. Reduced nitrogen (ammonium or casein hydrolysate or glutamine) will support continued somatic embryo development - i.e. stages beyond preglobular stage somatic embryos; nitrate will not and should therefore not be used alone to support continued embryo development after somatic embryo induction-initiation has begun. This too is an area that requires investigation in terms of permissive metabolism (Smith and Krikorian, 1989).

No doubt there are many more controlling factors similar to pH and type of nitrogen that will be encountered (cf. Krikorian, 2000).

Thus, the need to study the control mechanisms in this system still provides a wide range of opportunities to study effects on plant growth of distinctive environments such as that of Space, and on Earth provides a much-needed tool to improve responses in hitherto seemingly ‘non-embryogenic’ systems that have usually been described as being too "recalcitrant" or "fastidious" in their requirements to be induced to be embryogenic, i.e. express their embryogenic potential. Higher plant biotechnology is dependent upon reliable means to manipulate and manage developing plant cells in vitro and knowledge from such studies should go far to providing a better understanding of what controls expression of morphogenetic potential at various stages of the culture process–ranging from the primary explant to sustained subculture.

The role of somatic embryogenesis biotechnology as it relates to higher plants in the operation and management of a Controlled Ecological Life Support System (CELSS) will be apparent. An important point here is that opportunities to disclose new control mechanisms can emerge in the course of studies that are not necessarily directed towards a specific goal.

VIII. Emergence of a ‘New’ Perspectives on Somatic Embryogenesis

At Stony Brook, we made significant progress towards developing procedures for somatic embryogenesis in Space using daylily and carrot as models. In the course of this work we (1) developed and refined exquisitely sensitive systems; (2) increasingly appreciated that our long standing, healthy skepticism of many of the nominal truisms associated with what has come to be called "tissue culture technology" was totally justified.

Specifically, in the process of developing and defining our embryogenic cultures, we advanced to conceptualizing our results in a framework of either fostering or limiting of embryogenic progression at key points in the process. This amounted to providing of, or avoidance of what are normally thought of as stresses or insults at what we preferred to call "phenocritically sensitive or vulnerable" stages of differentiation and development. Significantly, our work was largely done in the context of developing sustainable cultures for Spaceflight purposes, extending their performance potential through many days in states of "suspended animation" in anticipation of Spaceflight initiation, and overcoming normally severe limitations to progression of free embryogenic cells in predominantly liquid environments, in contrast to progression in/on semi-solid (Smith and Krikorian, 1992; Krikorian, 1999).

Our focus on the smallest of totipotent cell units disclosed an essential and unappreciated feature of the somatic embryogenic process. The should provide a much-needed stimulus for re-directing current cell biological and molecular work (Komamine et al., 1992; Pennell et al., 1992; Mordhorst et al., 1998). Stated in its barest bones essential, the ‘new’ perspective is this: inefficiencies or recalcitrance of a system in the context of plant cell and tissue culture biotechnology should be viewed in a cell biological and developmental context as failed responses due to stresses and inappropriate environmental conditions limiting the progression of embryo development. The smaller and less developed advanced the unit, the greater the vulnerability to stress. This elegantly simple and precise focus on what is happening in growing, developing ‘embryogenic cell cultures’, again more precisely developing somatic embryos, should allow detailed investigation at a number of levels going from the single cell

stage to few cell stage to the multicellular level. It offers a way of studying what stress ‘really’ is so far as an embryogenic system is concerned (Krikorian, 1996d).

My early views on somatic embryogenesis in Space and establishing whether polarity could be established in low G have been given above. But that represents a good example of trying to use a system to answer a very basic question without first fully understanding the constraints of the system. It was nominally a well-established fact of life that totipotent cells which were morphogenetically competent could be induced to embark upon a course of embryogenic development. It was only a number of years later that in the course of developing ever-improved somatic embryo systems for use in Space experimentation that we showed in the Stony Brook laboratory that embryogenic cultures of daylily, indeed probably all plants, are already determined in the primary culture stage, well before a culture is perpetuated through subculture. The fact that cannot be over-emphasized, is that maintenance culture conditions ‘simply’ perpetuate a determined state in the form of a proliferative collection of initials limited in their development but undergoing what might be termed a ‘forced regenerative polyembryony’. Under these conditions, the initials do not progress beyond the first few divisions before the newly formed cells ‘separate’ (that is, cells within a developing embryo are shifted out of their normal position, ultimately leading to a detachment or fragmentation of cells and groups of cells from the embryogenic unit). A kind of repetitive embryogenesis is thus fostered and variously-sized polyembryonic fragments are produced. Regeneration is dependent on a permissive environment. So far as suspensions are concerned, somatic embryos are formed only as a consequence of the ‘direct’ development or advancement of pre-existing somatic embryo initials. Proembryogenic masses (PEMs) do not exist according to their more usual, classical as it were, definition (Thorpe, 1995). They are more accurately described as clusters of budding somatic embryo initials with varying capacity for development or as polyembryonic fragments of somatic tissue. All this emphasizes that established embryogenic suspensions cannot, by their very nature, constitute model systems for the study of the induction of somatic embryogenesis since these events would have already occurred (Krikorian, 2000).

The new facts which we have found, and their potential to redirect perspective and experiment planning, should assist scientists and plant tissue culturists in their efforts to generate and better control embryogenic systems. The finding that totipotent cells in suspension are already somatic embryos or zygote-equivalents and not ‘undifferentiated’ cells needing to be induced to the embryogenic state by any of a variety of ill-defined or empiric means proves that the long existence of a common belief is by no means infallible evidence of its correctness. It is proverbial that habit dies hard, and the judicial attitude is far from universal. From the outset, the very discovery of somatic embryogenesis has had a certain ambivalence associated with it. For many years the field has been fraught with many uncertainties and considerable empiricism, even failure to recognize what was actually happening in the cultures being worked with (Krikorian and Simola, 1999).

Again, while none of the above was directly related to Space biology, the findings from our research emerged in the course of attempting to develop and streamline procedures for testing in Space. In fact there had been, one might say, a preoccupation of trying to work with absolutely minimum units—‘free’ cells. I feel fortunate to have had the instigation to view the systems we worked on from a fresh perspective that fostered a particular mind-set and thus opened our eyes, so to speak, to unconventional interpretations as to what was needed for a well-controlled and manageable experiment. (Admittedly this all took a fair amount of time, some might say too long a period. The thesis of my last Graduate Student, Dr. Joel

Weidenfeld contains all the details and reference may be made to that pending full publication of the work, Weidenfeld, 2000.)

IX. Phenotype is Affected in Unexpected Ways by Environment: The Case of Ethylene and Morphology of Cultured Tissues

In the case of aseptically cultured daylily shoots, use of ancymidol (α-cyclopropyl-α-[p-methoxy-phenyl]-5-pyrimidine methyl alcohol) has shown that the compound has significant value in keeping plants short, with an optimum somewhere between 3 and 10 mg/liter. Growth has been repeatedly tested and shown to be suppressed over a 10 week period but normal growth resumes upon return to ancymidol-free medium. This strategy has had considerable value in enabling us to handle propagules of daylily more efficiently, especially in those situations where the capacity of a growing environment (vessel) in accommodating a large plant is limited. Cultures can be kept short, the ancymidol can be removed, the plant resumes 'normal' growth and the test conditions can be imposed.

The role of ethylene gas in maintaining and initiating transition to mature phenotype in daylily was discovered in the course of studies aimed at evaluating the effect of ethylene gas on growth of cultures in a sealed environment. It transpired that a phase change, typified by a very obvious change in phenotype, was effected by the presence of ethylene at a specific level. The phase change obtained led to a phenotype that is typical of the mature plant (Smith et al., 1989). It will be appreciated that a major problem in cloning operations of various plants by in vitro means is that they are frequently juvenile and require a period of growth (that is empirically determined) before they function as the adolescent or mature phenotype (Vasil and Thorpe, 1994; Soh and Bhojwani, 1999). In materials that are important in certain biotechnologies and agriculture, a better understanding of phase change and transition from juvenility to maturity is required before proper utilization of the given technology may be implemented.

Understandably, by far the most attention has been given to crystallizable chemicals and growth regulators as they apply to controlling mechanisms of higher plant growth but gases can play important roles as well (Kanellis et al., 1997). Since better understanding of closed or controlled environments in association with plant growth was, and is still needed, it was believed that this kind of activity would serve multiple purposes.

Again, chance or serendipity helped to shed some light on an important problem. Populations of miniature plantlets with fan-shaped growth form had been encountered a number of times in suspension-derived and suspension-derived but protoplast-generated embryogenic cells placed on semi-solid media. There was several particularly noteworthy features about these miniature, mature forms. All plantlets originating from a given stock or common culture did not follow one or other growth habit. Thus, we had no reason to believe that there was anything inherent within a given cell line or culture or its prior origin in terms of whether it came from cells or protoplasts, for obviously pre-disposing it to form fans. But, if several members of a population within a given culture vessel conspicuously showed the fan habit, close scrutiny showed that they all had the special feature. The same is true of juvenile populations. Mixtures of fans and juvenile forms were never seen. Because of asynchronous development of the plantlets in culture, especially on agar media, there is inevitably some variation in size within a culture vessel. Not only was there contrasting leaf morphology, but in the root system as well. In the juvenile state, the roots are fewer, thinner and longer. In the "mature" plantlets, the roots are more numerous, fleshy, thicker and more fibrous, much like

those of much older plants. Attempts to maintain the mature fan-shaped plantlets after removal from culture vessels were never successful. Plantlets consistently shown new leaf growth of the juvenile form and, when removed and planted in soil in due course, usually after a year or so, they embarked upon the course of growth that ultimately yielded fans. The possibility that the different growth forms were the result of localized micro-environmental influences within our growth chambers had been examined. Light and temperature were not responsible for the growth form differences. The hypothesis that “maturity-inducing/sustaining” substances(s) may have been produced and released into the medium by fan plantlets, and that mature growth might thus be prompted in juvenile plants was also been tested. When juvenile forms were placed aseptically on media in jars from which fan plantlets had been removed, and vice versa, the juvenile forms remained juvenile and the fan forms soon produced new juvenile growth. To our disappointment, juvenile forms never grew into fan forms under in vitro conditions--even when a substantial period of time had elapsed and one might normally expect fans. This may perhaps be due to depletion of nutrients and the dramatic slowing down of growth in cultures which have been maintained for many months on the same medium. (It does not follow, of course, that shoots with “mature” characteristics cannot arise in culture from "juvenile" meristems. Several daylily hybridizers, commercial growers and enthusiasts when questioned about ever having encountered the absence of, or a curtailment of the normal juvenile phase in seedling material stated, however, that they had never seen a mature form before its time.)

All tests involving opening of a culture vessel, even without subsequent removal of contents, resulted in the rapid reversal of the fan habit; juvenile forms remained unaffected. This suggested that a gas was being released to the environment whenever a jar was opened. (The occurrence of fans seemed to be correlated with the exceptionally tightly sealed jars.) Suspicions that a volatile or gaseous component of the environment might be involved were strengthened by a simple experiment. The mere loosening or "cracking" of the lid and re-tightening was sufficient to result in reversion of fans to the juvenile form. (Within a couple of days the reversal is complete.) To make a long story short, ethylene releasing substances, and inhibitors of ethylene production were tested and the change in phenotype was shown to be due to ethylene accumulation in the culture vessel (Smith et al., 1989).

Years ago, Hussey and Stacey (1981) called attention to an “ethylene morphology” in potato clones multiplied in sealed culture vessels. Nodes from plantlets generated in sealed vessels gave rise, however, to 'normal' shoots when transferred to loosely sealed vessels. In daylily, unlike potato, the form change involves size, it is clearly juvenile to mature, and it is reflected in root morphology as well. This all figures significantly in the improvement of vegetatively propagated plants (Abbott and Atkin, 1987).

The fact that the form change has never been shown to be permanent, that is that the mature fan shape reverts to the juvenile form, emphasizes that under the conditions tested, the form change is physiological in nature rather than epigenetic. Clearly, stabilization of the mature form could provide useful information on those events associated with determination phenomena. In any case, our work showed that the daylily shoot apex is not necessarily determined or programmed to produce leaf primordia in a fixed mode merely because it has reached a certain volume, size or age. (This was shown to be the case in Musa clones as well the apex of the raceme (male ‘floral’ bud)) was excised and grown in vitro (Krikorian et al., 1993, 1999).

Abnormalities of Plant Materials Exposed to Space, Cytogenetic Profiling and Somaclonal Variation

Yet another aspect of our work is intimately associated with observations made in Space-grown materials. Experiments first performed as mid-deck locker of the Space Shuttle on seedlings and later on aseptic propagules and still later on cell-culture-derived propagules early showed that chromosomal changes can occur that cannot be accounted for by the radiation environment; the measured radiation has been too low, even insignificant, to account for the changes.

Changes include chromosome rearrangements that are due to breaks at apparent "hot spots" that in turn lead to changes in ploidy and karyotype in ways that cannot as yet be duplicated on Earth. They may lead to permanently altered genotypes (Krikorian, 1996b).

All the evidence available indicates that perturbations in cell division are a major manifestation of stress (Krikorian, 1996d, 1999). The level of the stress effects are dependent on the particulars of the system, especially the 'developmental state and biology'. [Even so, we recognize that the same concerns of experimentation in Space vis à vis gravitational controls expressed above hold for radiation experiments in Space and that this important issue will also need to be resolved experimentally. Similarly, and unfortunately, Space tests which have disclosed that Spaceflight can have adverse effects on plant cell function such as division as evidenced by reduction in the level and fidelity of cell division were limited by necessarily imperfect controls onboard the Spacecraft. Recognizing this limitation brought on by unavailability of appropriate centrifuges in Space, disturbances ranging from slight to extreme - have been found at the level of the nucleus and chromosomes (Krikorian et al., 1992; Krikorian, 1996b, 1998; Conger et al., 1998).]
Binucleate cells in systems that are normally uninucleate, chromosomal deletions, translocations, aneusomaty, microchromosomes, bridges etc. (all changes that are not generally "removed" through diplontic selection in seed-producing species and certainly not in vegetatively propagated ones) as well as cells with massive chromosome fragmentation have been encountered in cells of Space-grown somatic embryos, Space-generated roots on tissue culture-derived plantlets, Space-generated roots on cloned seedlings and in roots of seedlings (Kann et al., 1991; Levine and Krikorian, 1996). Nevertheless, all test specimens, despite the fact that they were clonal or near-isogenic, have not always shown these effects although they were nominally in the same "hardware" or growing environment (Krikorian et al., 1992; Krikorian, 1999 and in process).
Careful scrutinizing of all the data (Krikorian, 1999) indicates that there are several interacting components as to the nature of the responses encountered. What we have learned about vulnerability or responsiveness of embryogenically competent free cells at specific "phenocritically sensitive" stages provided an appealing framework for hypothesis development and testing. It also has significance for understanding some problems generally recognized by cell and tissue culture workers–namely somaclonal variation.

Somaclonal variation is generally appreciated as brought on by a number of conditions. Less emphasis has been placed on its being brought on by various aspects of the culture process (cf. e.g. van Harten 1998). In connection with the need to be absolutely certain that materials exposed by us to the Space environment were chromosomally 'perfectly normal' prior to exposure, thus eliminating any potential criticism that results were due to imperfections in the materials exposed in the first instance, extra-ordinary precautions were taken to study the chromosomal profile during each stage of the culture process.

Our data from Space suggests that cells of species with large chromosomes and DNA content with variously located centromeres (e.g. not predominantly metacentric) (like daylily) show signs of considerable perturbation. Although data has been drawn from only a few species,

cells of polyploids with large chromosomes that are essentially metacentric (like hexaploid bread wheat) show very few disturbances (cf. Tripathy et al., 1996 for other results with wheat.) . The younger the somatic embryo in terms of its developmental progression, the more sensitive it is. The more advanced, the less (carrot and daylily). The more polyploid the system the more resistant to perturbation it seems to be but the higher the DNA level in the nucleus the more sensitive it seems to be (daylily 2n vs 4n). Cells of species with small chromosomes and low DNA content like mung bean and carrot show far fewer mitotic anomalies, sometimes even none. Samplings from intact, well-defined meristems like root tips of haplopappus (2n = 4) derived from germinated seedlings show fewer aberrations than those from de novo-generated root initials produced from aseptically-generated propagules or stem cuttings. Somatic embryos produced from small developing embryogenic initials tend to show more abnormalities than cells of materials that were more-developed or advanced at the time of their exposure (carrot and daylily). The smaller the responding embryogenic initial, the greater the vulnerability to perturbation and hence they show more "damage" in space (daylily). Embryogenic cells dispersed in a semi-solid medium tend to show less perturbation than those in liquid or on semi-solid media (carrot) (Krikorian, 1996c). These details and conceptual framework should go far towards enabling the resolution of some of the discrepancies that have been emphasized in the course of attempting a consistent interpretation of results from various bits of flight data (cf. e.g. Halstead and Dutcher, 1987). All this suggests that well-controlled experiments are now finally within sight of being performed provided we take into account "all" of the variables. Most of these variables were not apparent before we achieved the present level of sophistication in the initiation, development and analysis of our embryogenic systems using free cells.

If we interpret our "Space chromosome" results in a context of additive or synergistic response to stresses in combination with specific features of "biology" and "developmental stage", and wherein a level of stress sufficient to elicit stress symptoms is not reached under ground control conditions, the picture becomes significantly clearer. It becomes even easier to appreciate if we include the fact that behavior of fluids is dramatically altered in space . Because of the dominance of surface tension/molecular attraction, and absence of gravity-driven convection, fluids tend to deposit in unexpected or undesirable ways on plant surfaces and growth media, having many affects including greatly impeded air movement (Krikorian and Levine, 1991; Levine and Krikorian, 1992a,b). In this new context of level of developmental complexity and stage sensitivity, we were able to define the parameters of stress sensitivity and response, and to resolve its basis in a cell biological context. This should later facilitate better study in a more molecular context. It would be very attractive if Space effects could be explained on the basis of additive, known effects of stress since it means that one could avoid stress by a proper matching of objectives with plant material. This would be equivalent of employing proper counter-measures, as they are called. If we view our Space findings in this context, what is happening in the Space environment is that additive levels of stresses not normally experienced on Earth and which result from "Spaceflight conditions" additively or synergistically combine to foster the reaching of critical threshold levels of the stress-inducer, whatever its exact nature(s) may be. (It would still remain of course to be determined whether several things are going on in the Space environment, including synergism between radiation and microgravity, or responses to electromagnetic disturbances etc., or whether there are a "merely" a number of hitherto inadequately un- or underappreciated, hence uncontrolled, variables the effects of which are being manifested in the course of an experiment in Space.)
All the above will contribute to our understanding of growth regulation at various levels of complexity ranging from free cells in vitro to tissues to whole plants.

X. Final Analysis

A reader informed in plant tissue work will appreciate at once that developing and implementing plant tissue and cell culture technologies for use in Space research is sure to be no mean task. All plant-related technologies, are indeed, doubly difficult to put in place and implement, since the basic aspects need to be understood well enough to render a given system reliable and cost-effective.

Some years ago plant tissue cultures were examined, albeit superficially, from the perspective of possibly providing edible material for use in a Space setting (Byrne and Koch, 1962; Hildebrandt et al., 1963). Given the then state of the art, and in view of the food preference biases and economic constraints inherent in generation of unconventional food, even algae (Krauss, 1962; Lembi and Waaland, 1988), no-one has since then seriously given thought to direct use of a higher plant tissue culture as a food source. A consideration which had not then surfaced relates to the somewhat now routine use of low levels of growth substances (some of which, like the auxins, are active as herbicides in much higher doses) in initiating and maintaining higher plant cells and tissues in vitro. There is no doubt that the Food and Drug Administration-type regulations and health considerations nowadays would preclude direct consumption of tissue cultures so grown as human food because of the potential of cultures to retain and/or complex these growth substances. Eliminating potentially toxic residues by processing procedures could solve the problem but would add yet one more detail to be contended with. Even so, one should at least mention that there are some tissue culture and molecular biology strategies on the horizon which could well eliminate this drawback (Smith and Krikorian, 1989, 1990a). From the perspective of what technology is currently available or likely to become available in the moderately near-term, it seems likely that the most reasonable position for various national Space agencies is to view tissue culture--more precisely that aspect of it which deals with multiplication from pre-formed or organized starting materials--only from the viewpoint of a potential management tool. Micropropagation, as it is called, does have some substantial merit in a Controlled Ecological Life Support System setting. Not unreasonable uses of plant tissue culture for food production could even include such things as tomato fruits being generated from flowers produced from thin layers grown in vitro (Compton and Veilleux, 1991, 1992).

The scope of information currently available renders brief analysis of the systems presently available difficult. Table 3 attempts to summarize the kinds of activities that tissue culture techniques can facilitate. Near term, intermediate and long range implications are provided.

Table 3: Range of Activities that Currently Available Tissue Culture Techniques Can Facilitate

RESEARCH WITH POTENTIAL FOR NEAR TERM IMPACT
Culture techniques can facilitate:
- Rapid multiplication of select specimens
- Elimination of virus and specific pathogens
- Virus indexing
- Germplasm introduction and evaluation
- Germplasm collection, preservation and management
- Production of polyploids, haploids, somaclonal variants for new crop production and use in breeding, etc.
- Elimination of certain breeding barriers
  - . in vitro fertilization in ovulo
  - . embryo rescue and/or storage
  - . androgenesis
  - . gynogenesis

RESEARCH WITH INTERMEDIATE IMPACT

All the above in more recalcitrant species, plus

Selection for complex traits such as tolerance to stress

. biotic - diseases and pests

. abiotic – temperature, salt, herbicides

In vitro mutation breeding

Cryopreservation

RESEARCH WITH LONG RANGE IMPLICATIONS

All the above in still more recalcitrant species, plus

Genetic engineering

. transformation by selectable genes etc.

. organelle transfer

. wide crosses-somatic hybridization

Understanding controls in developmental and physiological processes

Since no-one can predict the time scale for the impacts listed, suffice it to say that research developments could in fact change significantly the ordering presented in Table 3. A fact that will readily be obvious is, however, that "real genetic engineering," that is bona fide, substantive and controlled manipulation of the higher plant genome for anything other than single-gene traits (i.e. polygenic traits) by recombinant DNA techniques is, in my opinion at least, some time off in the future. Also, it should be stressed that few informed investigators view any of the methods as providing directly materials which are useful as products ready for introduction into the agricultural or horticultural "pipeline." Instead, and especially in those cases where tissue culture-derived or "genetically-engineered" lines are envisioned, they are more properly viewed as the starting point for further genetic manipulation via more "conventional" plant breeding technologies. These processes will shorten the time needed for implementing change in traits etc. but the whole business will more a matter of degree than in kind.

However, be all that as it may, even a casual examination of Table 4 will disclose the range of benefits that could accrue to a Controlled Ecological Life Support System program. The question therefore becomes reduced to the level of commitment to bring about feasibility. Table 4 provides an overview of some of the advantages and disadvantages of in vitro systems for Space research and development. Like many other tasks which are specific to Space agency needs, the systems would, for the most part, have to be developed for the given purpose or activity mode.

Obviously, there are some exceptions to this statement. For instance, potato can easily be micropropagated, even by the uninitiated, on a simple, hormone-free medium using a very simple procedure (cf. Espinosa et al., 1984). Wheeler et al., (1986) have used the method to initiate potato plants for their Space-related work. One can readily envision use of such a system to manage activities in Space, provided the detailed parameters for plant life support are worked out.

Cryopreservation could likewise provide, especially in the context of long duration missions, a management potential for relatively efficient germ plasm storage. Unfortunately, cryopreservation as it is currently carried out normally does not provide the high degree of reliability that would be warranted in these cases (cf. Kartha and Engelmann, 1994; Benson, 1995). And, long-term storage of germplasm in vitro without these special precautions is fraught with difficulties although one might well be surprised with the stability that can be achieved without them (Sayavedra-Soto et al., 2000).

Although many of the problems that have to be dealt with are still research-problems, many others are ripe for development. Fine tuning development problems are still to be achieved but there is not a very big gap between initiating a plant tissue culture program which is highly predictable and ultimately what one would call a "process technology", and a "product technology". While these are still largely explorative (cf. Heinstein and Emery, 1988) the time has come to move forward with more confidence.

The encouraging aspect should be that one can predict with confidence that sooner or later all of the items in Table 3 will be possible. On the other hand, and as pointed out in Table 4 below (under "disadvantages") gaining access to the advantages of these activities will not be a casual or facile strategy.

Table 4: Excised Organ, Tissue, Cell and Protoplast Systems in a Space Setting

Advantages

1. Offers a broad range of capabilities, especially access to the controlled level of prior differentiation, growth and development, depending on the system adopted.
2. Once adopted, "tissue cultures" provide a standardized way of acquiring information - e.g. in flight acquisition of engineering data for plant growth systems etc.
3. Offer several options for clean "management" of biomaterials. Enables a "Modular Approach" to both basic and applied research.
4. Tissue culture systems provide the necessary interface between molecular biology and bio-engineering and these will ultimately permit a fuller understanding of plant development, growth, productivity etc., so crucial to a effort.

Disadvantages

1. Labor-intensive as “tissue culture” is generally and currently practiced. But effort beginning to be automated (cf. Holdgate and Zandvoort, 1992).
2. Development of experimental material is not a casual activity. It is a process not an event.
3. All species are not yet equally amenable to the full range of manipulations. Technologies, if they are to be generally applicable, obviously cannot be restricted to only certain species.

Similarly, it will be understood that conventional breeding or at least an awareness of what is available from traditional breeders will also have a role to play in a Controlled Ecological Life Support System effort. Plants that are smaller or "miniaturized" and hence require smaller space for growth, have shorter life cycles or specific stages of development, or have features such as low light or other specific physiological needs will obviously be useful in research.

XI. Outlook

Modest but realistic plans should be encouraged by the various Space agencies as soon as possible to develop tissue culture capabilities so that carefully orchestrated Controlled Ecological Life Support System-specific “tissue culture” work can be undertaken. In each instance, a detailed and thoughtful plan could be delineated within the framework of a sustained, long-term effort.

In those cases where specific pathogen-free or indexed propagules are seen to be of direct benefit, micropropagation should be undertaken and integrated. It will be important to start off modestly--e.g. with potato nodal or similar cuttings and to analyze systematically and at each step along the way the benefits that accrue from the use of in vitro methods as a tool.

Because tissue cultures are small, compact, relatively well controlled, and can be grown in large numbers, tissue culture capability provides an opportunity to select plants for use in growth chamber experiments. The idea here is that in vitro systems grown in modes conducive to eliciting change (so-called culture-associated or somaclonal variation), could well provide a convenient means of identifying plantlets or plants that can cope more efficiently within a given chamber or closed environment. Since there will be a long term need to determine the various levels of flexibility and/or constraints in growing and managing plants in the Space environment, such tissue culture-generated plants could play an important role in the operations. Certainly, in seeking an answer as to how to get a design for a "test bed" or facilities for model testing, one can use cultured tissues in addition to seed-generated plantlets. One major advantage that cultured systems afford is that by being miniaturized they

can facilitate obtaining answers to questions on such matters as: How do you provide the needed support for plants in Space?; What is the photosynthetic capacity of plants in Space?; Will plants produce acceptable yields in Space? etc. (cf. e.g. Bugbee, 1999).

As in all efforts, success with tissue cultures will depend on:

- -Investigators and support personnel with adequate scientific background and training
- -Financial resources
- -A bureaucracy sympathetic to research with a "lead-time" to "development" and administrators able to facilitate implementation of programs
- -Recognition not only of capabilities but limitations of "tissue culture" techniques
- -Teamwork at individual, institutional, local, national and international level
- -Maximum integration with other disciplines
- -Frequent and open communication

From the perspective of more traditional breeding, the most reasonable recommendation in the initial stages of such work would be that no special attempt be made to incorporate breeding -whether mutational (van Harten, 1998) or conventional (Baker, 1986;Richards, 1997)- directly into such programs. Instead, as needs arise or are anticipated, specific investigators or experts on a given species could be sought out with the view of ascertaining what "amenable" Space-biology plant germ plasm already exists, or what would be required to generate or "design" a variety or form that could serve a stated objective more effectively. In all this, however, it will be apparent that there will be trade-offs. The adage that "one is constantly seeking models which are 'models' for something other than themselves" should constantly be kept in mind.

References

Abbott, A.J. and R.K. Atkin, (Eds.) (1987): Improving Vegetatively Propagated Crops. Academic Press, London.

Arditti, J. and A.D. Krikorian (1996): Orchid micropropagation: the path from laboratory to commercialization and an account of several unappreciated investigators. Botanical Journal of the Linnean Society 122, 185-241.

Bajaj, Y.P.S. (Ed.) (2000): Biotechnology in Agriculture and Forestry. 44. Transgenic Trees. Springer-Verlag, Berlin.

Baker, R. J. (1986): Selection Indices in Plant Breeding. CRC Press, Boca Raton, FL.

Barlow, P.W. (1995): Gravity perception in plants: a multiplicity of systems derived by evolution? Plant, Cell and Environment 18, 951-962.

Bayvel, L. and Z. Orzechowski (1993): Liquid Atomization. Taylor & Francis. Washington, D.C.

Benedikt, E.T. (Ed.) (1960): Weightlessness- Physical Phenomena and Biological Effects. Plenum Press, NY.

Benson, E.E. (1995): Cryopreservation of shoot-tips and meristems. Pages 121-132. In: Cryopreservation and Freeze-drying Protocols, (J. G. Day and M. R. McLellan, eds.), Methods in Molecular Biology 38. Humana Press, Totawa, NJ.

Brown, A.H. (1996): Gravity related features of plant growth behavior studied with rotating machines. Journal of Gravitational Physiology 31, 69-74.

Bugbee, B. (1999): Engineering plants for spaceflight environments. American Society for Gravitational and Space Biology Bulletin. 12, 67-74.

Byrne, A.F. and R.B. Koch (1962): Food production by submerged culture of plant tissue cells. Science 135, 215-216.

Claassen, D.E. and B.S. Spooner (1994): Impact of altered gravity on aspects of cell biology. International Review of Cell Biology 156, 301-373.

Cogoli, A. and F.K. Gmünder (1991): Gravity effects on single cells. Advances in Space Biology in Medicine 1, 183-248.

Cogoli, A. and A. Tschopp (1982): Biotechnology in space laboratories. Advances in Biochemical Engineering 22, 1-50.

Compton, M.E. and R.E.Veilleux (1991): Shoot, root and flower morphogenesis on tomato inflorescence explants. Plant Cell, Tissue and Organ Culture 24, 223-231.

Compton, M.E. and R.E. Veilleux (1992): Thin cell layer morphogenesis. Horticulture Reviews 14, 239-264.

Conger, B.V., Z. Tomaszewski Jr., J.K. Macdaniels and A. Vasilenko (1998): Spaceflight reduces somatic embryogenesis in orchardgrass (Poaceae). Plant, Cell and Environment 21, 1197-1203.

Corey, K.A. and R.M. Wheeler (1992): Gas exchange in NASA's biomass production chamber. BioScience 42, 503-509.

Digby, J. and R.D. Firn (1995): The gravitropic set-point angle (GSA): the identification of an important developmentally controlled variable governing plant architecture. Plant, Cell and Environment 18, 1434-1440.

Doran, P.M. (1993): Design of reactors for plant cells and organs. Advances in Biochemical Engineering Biotechnology 48, 115-168.

Durzan, D.J. (2000): Metabolic engineering of plant cells in a Space environment. Biotechnology and Genetic Engineering Reviews 17, 349-383.

Dutcher, F. R., Hess, E. L. and Halstead, T. W. 1994. Progress in plant research in space. Advances in Space Research 14, 159-171.

Espinoza, N., R. Estrada, P. Tovar, J. Bryan and J.H. Dodds (1984): Tissue Culture Micropropagation, Conservation, and Export of Potato Germplasm. International Potato Center (CIP). Lima, Peru.

Greenberg, R. and B.R. Tufts (2001): Infecting other worlds. American Scientist 89, 296-299.

Goins, G.D., H.G. Levine, C.L. Mackowiak, R.M. Wheeler, J.D. Carr and D.W. Ming (2000): Comparison studies of candidate nutrient delivery systems for plant cultivation in Space. SAE (Society of Automotive Engineers Technical Paper Series 972304) 7 pages.

Halstead, T.W. and F.R. Dutcher (1987): Plants in space. Annual Review of Plant Physiology 38, 317-345.

Heinstein, P. and A. Emery (1988): Processes with plant cell cultures. In: Biotechnology (H.-J. Rehm and G. Reed, eds.), Vol. 6b, pp. 213-248. VCH, NY.

Hildebrandt, A.C., J.C. Wilmar, H. Johns and A.J. Riker (1963): Growth of edible chlorophyllous plant tissues in vitro. American Journal of Botany 50, 248-254.

Holdgate, D.P. and E.A. Zandvoort (1992): Automated micropropagation and the application of a laser beam for cutting. Pages 297-311. In: Transplant Production Systems : Proceedings of the International Symposium on Transplant Production Systems, Yokokama, Japan, 21-26 July 1992; (K. Kurata and T. Kozai, eds.). Kluwer Academic Publishers, Dordrecht.

Hoshizaki, T. (1973): Influence of gravitational forces on plants. Environmental Biology and Medicine 2, 47-79.

Hussey, G. and N.J. Stacey (1981): In vitro propagation of potato (Solanum tuberosum L.). Annals of Botany 48, 787-796.

Kanellis, A.K. et al. (Eds.) (1997): Biology and Biotechnology of the Plant Hormone Ethylene. Kluwer Academic, Dordrecht and Boston.

Kann, R.P., S.A. O'Connor, H.G. Levine, and A:D. Krikorian (1991): Generation and multiplication of plantlets from callus derived from capitulum of Haplopappus gracilis (Nutt.) Gray and their karyotype analysis. Plant Science 75, 245-255.

Kartha, K.K. and F. Engelmann (1994): Cryopreservation and germplasm storage. In: Plant Tissue Culture, (I. K. Vasil and T.A. Thorpe, eds.), pp. 194-230; Kluwer Academic Publishers, Dordrecht, Netherlands.

Keefe, J.R. and A.D. Krikorian (1983): Gravitational biology on the space platform. Paper presented in the 13th Intersociety Conference on Environmental Systems. 11-13 July 1983, San Francisco, CA. SAE (Society of Automotive Engineers Technical Paper Series 831133) 23 pages.

Kliss, M. and R.D. MacElroy (1990): Salad machine: a vegetal production unit for long duration missions. Paper presented in the 20th Intersociety Conference on Environmental Systems 9-12 July, Williamsburg, Va. SAE (Society of Automotive Engineers Technical Paper Series 901280) 23 pages.

Komamine, A., R. Kawahara, M. Matsumoto, S. Sunabori, T. Toya, A. Fujiwara, M. Tsukahara, J. Smith, M. Ito, H. Fukuda, K. Nomura and T. Fujimura (1992): Mechanisms of somatic embryogenesis in cell cultures: Physiology, Biochemistry, and Molecular Biology. In Vitro Cell Dev. Biol. 28, 11-14.

Kozai, T., K. Fujiwara, M. Hayashi and J. Aitken-Christie (1992): The in vitro environment and its control in micropropagation, In: Transplant Production Systems. (K. Kurata and T. Kozai, eds.) pp. 247-282. Kluwer Academic Publishers, Dordrecht and Boston.

Krauss, R.W. (1962): Mass culture of algae for food and other organic compounds. American Journal of Botany 49, 425-435.

Krikorian, A.D. (1991): Embryogenic plant cells in microgravity. American Society for Gravitational and Space Biology Bulletin 4, 65-72.

Krikorian, A.D. (1996a): Strategies for “minimal growth maintenance” of cell cultures: A perspective on management for extended duration experimentation in the microgravity environment of a space station. Botanical Review 62, 41-108.

Krikorian, A.D. (1996b): Embryogenic somatic cell cultures of daylily (Hemerocallis): A system to probe spaceflight-associated mitotic disturbances. In: Plants in Space Biology (H. Suge, ed.), pp. 111-126. Institute of Genetic Ecology, Tohoku University, Sendai.

Krikorian, A.D. (1996c): Gravity and the stability of the differentiated state of plant somatic embryos (PEMBSIS). In: IML-2 Final Report of IML-2 Experiments, pp. 163-178. Space Experiment Department, Office of Space Utilization Systems, National Space Development Agency of Japan. NASDA-TMR-960004.

Krikorian A.D. (1996d): Space stress and genome shock in developing plant cells. Physiologia Plantarum 98, 901-908.

Krikorian, A.D. (1997): Commercializing micropropagated ferns. Singapore National Academy of Sciences (SNAS) Journal 24/24, 84-96.

Krikorian, A.D. (1998): Plants and somatic embryos in Space: What have we learned? American Society for Gravitational and Space Biology Bulletin 11(no.2), 5-14.

Krikorian, A.D. (1999): Somatic embryos of daylily in space. Advances in Space Research (32nd COSPAR Scientific Assembly, Nagoya, Japan) 23, 1987-1997.

Krikorian, A.D. (2000): Historical insights into some contemporary problems in somatic embryogenesis. In: Somatic Embryogenesis in Plants. (S. Mohan Jain, P. K. Gupta and R. J. Newton, eds.) Volume 6, 17-49. Kluwer Academic Publishers, Dordrecht.

Krikorian, A.D., F. R. Dutcher, C.E. Quinn and F.C. Steward (1981): Growth and development of cultured carrot cells and embryos under space flight conditions. In: Advances in Space Research Volume l, 117-127. W.R. Holmquist (Ed.). Pergamon Press, Oxford, NY.

Krikorian, A.D., H. Irizarry, S.S. Cronauer-Mitra and E. Rivera (1993): Clonal fidelity and variation in plantain (Musa AAB) regenerated from vegetative stem and floral axis tips in vitro. Annals of Botany 71, 519-535.

Krikorian, A.D., H. Irizarry, R. Goenaga, M.E. Scott and B.E.L. Lockhart (1999): Stability in plant and bunch traits of a French-type dwarf plantain micropropagated in vitro from the floral stem tip and five lateral corm tips of a single mother plant: Good news on the tissue culture and bad news on banana streak virus. Scientia Horticulturae 81, 159-177.

Krikorian, A.D. and H.G. Levine (1991): Development and growth in Space. Pages 491-555. In: Plant Physiology: A Treatise (R.G.S. Bidwell and F.C. Steward, eds.) Vol. X. Academic Press, NY.

Krikorian, A.D., H.G. Levine, R.P. Kann and S.A. O'Connor (l992): Effects of spaceflight on growth and cell division in higher plants. In: Advances in Space Biology and Medicine, (S.L. Bonting, ed.), Vol 2, pp. 181-209. JAI Press, Greenwich, CT.

Krikorian, A.D. and L.K. Simola (1999): Totipotency, somatic embryogenesis and Harry Waris (1893-1973). Physiologia Plantarum 105, 348-345.

Krikorian, A.D. and D.L. Smith (l992): Somatic embryogenesis in carrot (Daucus carota. In: Plant Tissue Culture Manual: Fundamentals and Application, (K. Lindsey, ed.), pp. PTCM-A9 1-32, Kluwer Academic Publishers, Dordrecht.

Lembi, C.A. and J.R. Waaland (1988): Algae and Human Affairs. Cambridge University Press, Cambridge.

Levine, H.G. and A:D. Krikorian (l992a): Chromosomes and plant cell division in space: Environmental conditions and experimental details. Advances in Space Research 12, 73-82.

Levine, H.G. and A.D. Krikorian (1992b): Shoot growth in aseptically cultivated daylily and haplopappus plantlets after a 5-day spaceflight. Physiologia Plantarum 86, 349-359.

Levine, H.G. and A.D. Krikorian (l996): Root growth in aseptically cultivated plantlets of Haplopappus gracilis after a five day spaceflight. Journal of Gravitational Physiology 31, 17-27.

Lindsey, K. (1992): Plant Tissue Culture Manual. Kluwer Academic Publishers, Dordrecht.

Markert, C.L. and A.D. Krikorian (1993): Life: Origin and evolution on earth; How can we escape? In: "A Scientific Role for Space Station Freedom - How Space Platforms Can Serve Research in Life Sciences". AIAA-92-1348. Presented at a Symposium at AIAA [American Institute of Aeronautics and Astronautics Space Programs and Technologies Conference] Huntsville, Alabama 24 March 1992. NASA Technical Memorandum 4502, pp. 35-39.

Meyerowitz, E. M. (Ed.) (1994): Arabidopsis. Monograph 27. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mitchell, C.A. (1993): The role of bioregenerative life-support systems in a manned future of space. Transactions of the Kansas Academy of Sciences 96, 87-92.

Mordhorst, A.P., K.J. Voerman, M.V. Hartog, E:A. Meijer, J. van Went, M. Koorneeff and S. De Vries (1998): Somatic embryogenesis in Arabidopsis thaliana is facilitated by mutations in genes repressing meristematic cell divisions. Genetics 149, 549-563.

Musgrave, M.E., A. Kuang and S.W. Matthews (1997): Plant reproduction during spaceflight: importance of the gaseous environment. Planta 203, S177-S-184.

Nechitailo, G.S. and A.L. Mashinsky (1993): Space Biology. Studies at Orbital Stations. Translated by N. Lyubinov. Mir Publishers, Moscow.

Neumann, K.-H. (1995): Pflanzliche Zell- und Gewebekulturen. Eugen Ulmer Verlag, Stuttgart.

Nielsen, S.S., M.A. Belury, K.P. Nickel and C.A. Mitchell (1996): Plant nutrient composition altered with controlled environments for future space life-support systems. In: Progress in New Crops, (J. Janick, ed.), pp. 624-632, ASHS Press, Arlington, VA.

Pennell, R.I., S. De Vries and K. Roberts (1992): Identification of a transitional state in the developmental pathway to carrot somatic embryogenesis. Journal of Cell Biology 119, 1371-1380.

Pirie, N.W. (1980): Space victualling. Endeavour, N.S. 4, 74-77.

Prince, C.L., V. Bringi and M.L. Shuler (1991): Convective mass transfer in large porous biocatalysts: plant organ cultures. Biotechnology Progress 7, 195-199.

Raghavan, V. (1997): Molecular Embryology of Flowering Plants. Cambridge University Press, Cambridge and NY.

Raghavan, V. (2000): Developmental Biology of Flowering Plants. Springer Verlag, NY.

Ream, W. and S.B. Gelvin (1996): Crown gall: Advances in Understanding Interkingdom Transfer. American Phytopathological Society, St. Paul, MN.

Reynolds, P.H.S. (Ed.) (1999): Inducible gene Expression in Plants. CABI Publishing, Oxford and NY.

Richards, A.J. (1997): Plant Breeding Systems. 2nd ed. Stanley Thomas, London.

Sachs, T. (1991): Pattern Formation in Plant Tissues. Cambridge University Press, Cambridge.

Sack, F.D. (1991): Perception of gravity by plants. International Review of Cytology 127, 193-252.

Sack, F.D. (1998): Autotropism, automorphogenesis and gravity. Physiologia Plantarum 102, 328-335.

Salisbury, F.B. (1991a): Lunar farming: Achieving maximum yield for the exploration of space. Hort. Science 26, 827-833.

Salisbury, F.B. (1991b): Some challenges in designing a Lunar, Martian, or microgravity CELSS. Acta Astronautica 27, 211-217.

Sayavedra-Soto, L., A.D. Krikorian and M. Reporter (2000): Long term stability of electromanipulated protoplasts of Glycine max var. Acme and a Catharanthus roseus mutant. Journal of Plant Physiology 156, 137-140.

Smith, D.L., K. Kelly and A.D. Krikorian (1989): Ethylene-associated phase change from juvenile to mature phenotype of daylily (Hemerocallis) in vitro. Physiologia Plantarum 76, 466-473.

Smith, D.L. and A.D. Krikorian (1989): Release of somatic embryogenic potential from excised zygotic embryos of carrot and maintenance of proembryonic cultures in hormone-free medium. American Journal of Botany 76, 1832-1843.

Smith, D.L. and A.D. Krikorian (1990a): pH Control of carrot somatic embryogenesis.. In: Progress in Plant Cellular and Molecular Biology. Proceeding's, VII International Congress on Plant Tissue and Cell Culture, (H.J.J. Nijkamp, L.H.W. Van der Plas and J. van Aartrijk, eds.), pp. 449-453, Kluwer Academic, Dordrecht, Netherlands.

Smith, D.L. and A.D. Krikorian (1990b): Low external pH replaces 2,4-D in maintaining and multiplying 2,4-D-initiated embryogenic cells of carrot. Physiologia Plantarum 80, 329-336.

Smith, D.L. and A.D. Krikorian (1991): Growth and maintenance of an embryogenic cell culture of daylily (Hemerocallis) on hormone-free medium. Annals of Botany 67, 443-449.

Smith, D.L. and A.D. Krikorian (l992): Low external pH prevents cell elongation but not multiplication of embryogenic carrot cells. Physiologia Plantarum 84, 495-501.

Soh, Woong-Young and S.S. Bhowjwani, (Eds.) (1999): Morphogenesis in Plant Tissue Cultures. Kluwer Academic Publishers, Dordrecht.

Thorpe, T.A. (Ed.) (1995): In Vitro Embryogenesis in Plants. Kluwer Scientific, Dordrecht and Boston.

Tripathy, B.C., C.S. Brown, H.G. Levine and A.D. Krikorian (1996): Growth and photosynthetic responses of wheat plants grown in Space. Plant Physiology 110, 801-806.

van Harten, A.M. (1998): Mutation Breeding. Theory and Practical Applications. Cambridge University Press, Cambridge.

Vasil, I.K. and T.A. Thorpe. (Eds.) (1994): Plant Tissue Culture. Kluwer Academic, Dordrecht.

Vroemen, C., S. De Vries and R. Quatrano (1999): Signalling in plant embryos during the establishment of the polar axis. Cell & Developmental Biology 10, 157-164.

Weidenfeld, J. (2000): Towards Defining a Functional System for Somatic Embryogenesis. Ph.D. Thesis, State University of New York at Stony Brook, Stony Brook, New York. 297 pages.

Wheeler, R.M., K.L. Steffen, T.W. Tibbitts and J.P. Palta (1986): Utilization of potatoes for life support systems II. The effect of temperature under 24-H and 12-H photoperiods. American Potato Journal 63, 639-647.

Wilson, P.E.G. and M.G. Hilton (1995): Plant cell bioreactors. Pages 413-439. In: Bioreactor System Design, (J.A. Asenjo and J. Merchuk, eds.), Marcell Dekker, NY.

Wilson, Z.A. (Ed.) (2000): Arabidopsis: A Practical Approach. Oxford University Press, Oxford.

Wolverton, B.C., R.C. McDonald and W.R. Duffer (1983): Microorganisms and higher plants for waste water treatment. Journal of Environmental Quality 12, 236-242.

Wolverton, B.C., R.C. McDonald and E.A. Watkins (1984): Foliage plants for removing indoor air pollutants from energy-efficient homes. Economic Botany 38, 223-228.

Zimmermann, U., R. Schnettler and K. Hannig (1988): Biotechnology in space: Potentials and perspectives.. In: Biotechnology. Vol. 6b. Special Microbial Processes, (H.- J. Rehm and G. Reed, eds.), pp. 639-672 VCH Verlagsgesellschaft, Weinheim.

Ayurvedic Medicines: Some Potential Plants for Medicine from India

Ashwani Kumar

Energy Plantation Demonstration project and Biotechnology Center,

Department of Botany, University of Rajasthan, Jaipur, 302004 (Rajasthan) India.

e-mail: msku4@hotmail.com

Abstract

With the changing pattern of life style most of the diseases are now becoming lifestyle diseases. The traditional systems of medicine based on ancient cultures are primarily concerned with building the body strength which can help in healing the ailments and these systems rely largely on the nature cure. The Ayurvedic system has described a large number of such medicines based on plants or plant product and the determination of their morphological and pharmacological or pharmacognostical characters can provide a better understanding of their active principles and mode of action.

Introduction

Contribution of the traditional medicine to human health in the 21st Century is of paramount importance. A meeting of the International Forum on Traditional Medicine held recently (1999) at theToyama Medical and Pharmaceutical University, Toyama, Japan, reviewed the potential of traditional medicines. WHO acting director Xhang emphasized that with the changing pattern of life style most of the diseases are now becoming life style diseases. Natural medicines improve the inner strength of the body. The use of traditional medical systems has attracted so much attention that an International Health Center has been opened in July in the Toyama prefecture (Province)

Some of the oldest traditional medical systems include Chinese, Ayurvedic, Unani, Japanese and recently added homeopathy and chiropathy that is also around 200 years old. The use of traditional medicine includes (i) medication by use of medicinal plant, minerals, animal material and (ii) non medication: acupuncture and yoga.

Complementary medication includes acupuncture, herbal treatment, manual, spiritual and dietary treatments.

Toyama hospital utilizes vast amount of Chinese, Japanese and Ayurvedic medicine. Detailed studies in the areas of pharmacognosy and pharmacology are under progress (Annual report, TMPU, Toyama). Besides this the Research Center for Ethnomedicines with its Museum of Materia Medica is one of its own kind in the world under Professor Watanabe. Dr. Komatsu provides a wealth of information for all scientists engaged in the field all over the world. This includes identification, molecular characterization at DNA sequence level, chemical characterization, biotransformations and studies on effects on microorganisms to direct application in the hospital. To give an entire description will be attempted in another paper. Here a brief attempt is made to identify common goals of research in India and Japan, with an objective to attract attention of workers to the great potential that the vast bio-diversity of the Indian subcontinent and the wealth of Ayurvedic literature has to offer for future development of traditional medicines. However detailed future investigations are needed in this area to exploit the unexplored or poorly explored plant materials.

These traditional medicines have found practical application at clinical level in TMPU and over hundred cases of fissure have been cured in the hospital using a special thread prepared from latex of Euphorbia spp., thor of India (Euphorbia sp) , haldi powder (*Curcuma longa)* and some herbal ingredients. The *Euphorbia* sp is a plant of the desertic region of India and different parts of the world. A large number of energy yielding desertic plants of India used in the Ayurvedic system have great potential as Ayurvedic medicine . Negative environmental effects of current agricultural practices, such as emission of greenhouse gases, nutrient leaching, decreased soil fertility, and erosion, may be reduced when traditional annual food crops are replaced by dedicated perennial energy crops and medicinal plants. As they are able to grow and produce valuable products under dessert conditions they have great potential for covering the global desert areas into green belts leading to environmental improvement on one hand and providing valuable Ayurvedic crude drugs in addition to supplementing the bio-energy resources as renewable fuels. However detailed studies on their pharmacognostical characterization and determination of chemical products obtained from them are lacking. Some of the investigations indicated their potential use in Human immuno deficiency (HIV) diseases (Hattori et al., 1995). Such bio-energy plants have not been explored in depth. Here an attempt shall be made to provide a brief outlook of the Indian scene and highlight some of the work being carried out at our place in Rajasthan along with the possible impact assessment for desertic plants for future research strategies.
Among the desert plants the value of *Aloe vera* (L.) was recognized more than 3000 years ago when the Egyptian and Greek civilizations used its extract for skin burns, cuts and wounds on the skin surface and found that it had a wonderful healing effects on the skin. It is claimed that even 3rd degree burns can be cured and healed by *Aloe vera*. The chemical compounds like Aloein, resins and a mixture of polysaccharides containing pectic acid are present. Modern investigations indicate that extracts of *Aloe vera* act on the dead epithelial cells of the skin, aiding their removal from the surface and stimulating

the growth of new cells. Thus Aloe is a great gift of traditional medicine for protecting the smooth skin of human beings especially when radiation damage has assumed an alarming situation due to stratospheric ozone depletion. Fresh juice of leaves are also used in liver and spleen troubles and also for eye troubles, found useful in X-ray burns, dermatitis, coetaneous and other skin disorders.

In India, Egypt and Sudan around 70 percent of the rural people use traditional medicine. Similar situation exists in a large number of developing countries. In India and China 60 percent of the people affected with cholera and malaria are treated with herbal medicines. In these countries the market for traditional medicines is US $ 500 million while Western type medicine account for only 300 million US $. In Singapore 50 percent and in Australia 60 percent of population uses alternative medicine. Around 17,000 herbal products are registered in these countries. In Belgium 40 percent contemporary but 84 percent home medicines and 74 percent acupuncture medicine is utilized. In France 50 percent of the people take advantage of complementary medicine. In Germany 10,000 to 13, 000 alternative medical practitioners are thriving well and 75 percent of them utilize complementary medicines. 77 percent of pain clinics utilize acupuncture. In UK 90 percent of the complementary medical practitioners utilize osteopathy and acupuncture. In US where in 1990 only 30 percent of the people were utilizing complementary medicines, it grew to 40 percent in 1997.

Ayurvedic system of Medicine

Ayurveda is an offshoot of Atharva veda written over 3000 thousand years ago. The Charak and Sushruta describe a large number of crude drugs and a large part of them has origin to plants. However though some part of it has been translated from Sanskrit to Japanese and the Japan Society of Ayurveda under Professor Dr. Namba is very active in this field. But many of the crude drugs described remain to be identified to its plant source in botanical terms and the Institute of Traditional Medicine is the prime center for understanding the nature and morphology of crude drugs of Ayurvedic origin and their identification to the plant level. The personal communication with Professor Watanabe and Dr. Komatsu during my stay at Wakan Yaku as visiting Professor has contributed to the stimulation of such studies back home and some of the important findings are presented here. During my stay here I have worked on Nepalese crude drugs with support and guidance from Dr. Komatsu and other members of this institute.

The basic philosophy of Ayurveda considers that man is an inseparable part of the universe. The human body, mind and spirit continuum is an integral whole and the individual is also linked to the family, society, environment and ultimately the universe. The definition of health is that " It is state of complete psychosomatic equilibrium. It does not mean only absence of diseases but a state in which the mind, senses and spirit are pleasant and active". That agrees with the definition of WHO "Health is a state of complete physical, mental and social well being and not merely the absence of disease or infirmity"

India with its varied climate, soils and agro-ecology possesses an immense plant diversity, with over 15,000 species of higher plants. Both our Indian civilization as well as our diverse tribal heritage have gone a long way in conserving the wild weedy species, native land races and primitive cultivars (Fig. 1). The Indian gene center is endowed with rich flora, especially with regard to several less known yet economically important plants, ca. 160 cultivar species of economic plants, plus 56 species of lesser known cultivated food plants. Further there are ca. 320 species of wild and weedy economic types (Paroda, 1979; Arora and Nayar, 1984 ; Kumar, 1998).

The unutilized and underutilized resources

Out of 2,50,000 plant species only 10,000 or so have been exploited during the course of human civilization. A large number of hydrocarbon yielding plants are able to grow under semi arid and arid conditions and they also produce valuable hydrocarbons (up to 30 percent of dry matter) which could be converted into petroleum-like substances and used as fossil fuel substitute. They are rich in triterpenoids which are constituent to important drugs against HIV.

The potential plants

Certain potential plants were selected and attempts were made to develop agro-technology for their large scale cultivation (Kumar et al, 1995, Kumar, 1998, Kumar, 2000). A 50 ha bio-energy and medicinal plants cultivation demonstration center has been established on the campus of the University of Rajasthan to conduct the experiments on large scale cultivation of selected plants with the objective of developing optimal conditions for their growth and productivity, besides conserving the bio-diversity. Plantation of laticiferous plants and desert plants can be carried out, it could also lead to reclamation of marginal land that has already been abandoned in developed as well as developing countries. India alone has over 144 million hectare of marginal land which is about half of the total geographical area of the country. Touched only marginally by the green revolution, Africa suffers not only a dramatic nutritional problem but also an equally serious and inter linked problem of energy. Increasing scarcity of fuelwood, desertification, lack of water, food and medicines, excessive urbanization are all closely interdependent and rich biodiversity in developing countries has remained unutilized and underutilized for want of proper investigations.

There are surely opportunities for biomass of the medicinal plants in the south as well as in the north in wet climates and in dry ones but they will respond to very different schemes and strategies. There is not going to be a single unique recipe, rather multiplicity of solutions depending on climate, soil, availability of land, traditions as well as social and economic conditions. Technological improvements should lower production costs but they are unlikely to obtain significantly higher yields, as chemical and energy inputs must be reduced. The transformation of biomass into useful energy products and medicinal

compounds may however involve onsite industrial operations that could absorb at least part of the surplus man power.

As far as research is concerned we are all aware of the important progress being made in agricultural biotechnology. Genetic engineering for example is increasingly applied to crop plants for improving resistance to pests and diseases and for providing more favorable crop composition. There is a whole universe of possibilities in the use of advanced biotechnology to improve plants and processes. The natural medicine from plants has enormous possibilities for new and more effective means for curing the modern day ailments.

Natural resource

Total land area of Rajasthan is 3,42,239 sq km out of which 45.25 percent is characterized as wasteland. Large portions of this land were productive at a given time and due to man made deforestation, cattle pressure, water and wind based soil erosion, improper water management, they have turned out to be wastelands. (Kotia and Kumar, 2001a). A detailed survey on the weeds on wastelands yielded valuable data about the first colonizers. Out of the total weeds around 50 having important medicinal values while others produce related compounds. These regions are rich in bio-diversity and weeds were collected from different regions of the developing wastelands. (Kotia and Kumar, 2001b) .
Some of the medicinally important plants of Rajasthan are listed by Ajanta and Kumar, (2001a) They include species listed in table 1:

Table1: List of Medicinal plants of Rajasthan.

Plant species:	Local name
1. *Asparagus racemosus*	Satavari
2. *Chlorophytum arundinaceum*	Safed musli
3. *Curculigo orchioides*	Kali Musali
4. *Solanum surattense*	Kantkari
5. *Boerhaavia diffusa*	Santhi,
6. *Hamidesmus indicus*	Anantmool
7. *Sida cordifolia*	Bala
8. *Holarrhena antidysenterica*	Indrajo
9. *Curcuma aromatica*	Vanhaldi
10. *Oroxylum indicum*	Shyonaka
11. *Balanites aegyptiaca*	Hingot
12. *Withania somnifera*	Ashwagandha
13. *Aegle marmelos*	Bael
14. *Cassia fistula*	Amaltas
15. *Gymnema sysvestre*	Gudmar
16. *Terminalia arjuna*	Arjuna
17. *Butea monosperma*	Palas

18. *Soymida febrifuga*	Rohan
19. *Woodfordia fruticosa*	Dhavri
20. *Tribulus terrestris*	Gokhru
21. *Pedalium murex*	Badagokhru
22. *Vitex negundo*	Negad
23. *Dyerophytum indicum*	Chhitral
24. *Plumbago zeylanicum*	Chitrak
25. *Plantago ovata*	Isabgol
26. *Colocynthes vulgaris*	Indrayan
27. *Adhathoda vasica*	Ardusta
28. *Allangium salvifolium*	Aankol
29. *Caesalpinnia bonducella*	Tas
30. *Jatropha curcas*	Ratanjot
31. *Eclipta alba*	Bhringraj
32. *Aloe barbadensis*	Gwarpatha
33. *Mucuna prutita*	Konch
34. *Terminalia bellerica*	Baheda
35. *Tamarindus indica*	Imli
36. *Azadirachta indica*	Neem
37. *Achyranthes aspera*	Aandhijhara
38. *Barleria cacrulea*	Bajrandantis
39. *Barleria cristata*.	Badradantip
40. *Barleria prinoitis*	Bajradantip.
41. *Ocimum americanum*	Bapchii
42. *Centella asiatica*	Brahmibuti
43. *Datura metel.*	Dhatura
44. *Convolvulus arvensis*	Haranpadi
45. *Evolvulus alsinoides*	Shankhpushpi
46. *Cassia occidentalis*	Kasaundi
47. *Urginea indica*	Kolikanda
48. *Andrographis paniculata*	Kalmegh
49. *Helicteres ispara*	Marorphali
50. *Tinospora cordifolia*	Nimgiloy

Calotropis procera (Ait.) R.Br. (Akanda, Alarka, Aak) : The plant is one of the important numbers of traditional herbal medicine in every home of India. Traditionally the leaves of aak are warmed and tied around any body organ in pain. It is practically useful in backache and in joint pains. Warm leaves also relieve from stomach ache if tied around. Inhalation of burnt leaf cures headache. The traditional folk healers use the milky latex of aak for several ailments. Leaf latex if applied on fresh cut, stops bleeding immediately. Recent investigations have found that the alkaloids calotropin, calotaxein and uskerin are stimulant to the heart. Flowers and roots are used in Ayurvedic medicine. The plant is anthelmintic, the ashes act as an expectorant. The leaves are applied hot to the abdomen to cure the pain inside. The flower is tonic, antisialagogue, used as appetizer and against stomach ache, and cures piles and asthma. Flowers are believed to have detergent properties so they are given in cholera. The fresh roots are used as a toothbrush and are considered by pathans to cure toothache.. Alarka is an

alternative tonic and diaphoretic, in large dose emetic. Root bark is useful for treating chronic cases of dyspepsia, flatulence, constipation, loss of appetite, indigestion and mucus in stools. Leaves are used against guinea worms. Flowers are useful in asthma. Seed oil is geriatric and tonic. Green copra is given in asthma. Plant is used in spleen complaints, rheumatism, epilepsy, hemiplegia, sores, and smallpox and protracted labor.

Calotropis gigantea R.Br. (Arka) : Arka is purgative, anthelmintic alexipharmic,; cures leprosy, ulcers, leucoderma, tumors, piles, diseases of spleen, liver and abdomen. Juice is anthelmintic and laxative; cures piles and kapha. Dried and powdered plant is taken with milk and acts as a good tonic. Action is similar to Digitalis on the heart. Root bark and juice have emetic, diaphoretic, alternative and purgative properties. It is used in dysentery and as a substitute for Ipecauantha. It is regarded as a great remedy in syphilitic afflictions and is called "Vegetable mercury". In intermittent fevers it is used as antiperiodic and diaphoretic. It cures asthma and syphlis. In form of paste it is applied to elephantiasis. Tincture of leaves is used in intermittent fevers. Latex is bitter, heating, oleagenous and irritant, used in combination with *Euphrobia neerifolia* as purgative. Flowers are sweet, bitter, digestive, tonic, stomachic, anthelmintic, analgesic, astringent; cure inflamations, tumours, kapha and are good in ascites.

Jatropha curcas Linn. (Vyagrairanda) : Juice of Vyagrairanda is a well known purgative and is useful in whitlow, convulsions, syphilis, neuralgia, dropsy, anasarca, pleurisy and pneumonia. Root bark is applied externally in rheumatism and is used in sores. Leaves are galactagogue, rubefacient, suppurative, insecticidal and are used in foul ulcers, tumors and scabies, given internally in jaundice. Leaves are locally applied to breasts to increase secretion of milk. Leaves warmed and rubbed with castor oil and applied to boils and abscesses have supportive effect. Decoction of leaves is against diarrhoea, useful in stomach-ache and cough and also used for gargle to strengthen gums. Fresh stems are used as toothbrush. Fresh viscid juice flowing from stem is employed to arrest bleeding or hemorrhage from wounds. Stem bark is used for wounds of animal bites. Fruits and seeds are anthelmintic, useful in chronic dysentery, urinary discharges, abdominal complaints, anaemia, biliousness, fistula, and diseases of heart. Seeds are acro-nacrotic, poisonous to human beings and cattle and used against warts and cancers and also to promote hair growth. Seeds and oil are purgative, more drastic than castor oil. Wood causes dermatitis. Drug is bitter, acrid, astringent and anthelmintic. It serves to cleanse the entire system through its purgative property. It is useful in chronic dysentery, thirst, abdominal complaints, biliousness, anemia, fistula, ulcer, and diseases of the heart and skin.

Croton tiglium Linn. (Jamaalagotta, Jayapala) : Jayapala seeds and oil are drastic purgative, diaphoretic, vasicant, vermifuge irritant, rubefaceint and cathartic. Its action is prompt. Croton oil when rubbed on skin acts as a rubefaceint and counter-irritant and vesicant. When administered internally it operates as a powerful hydrogue cathartic. It is found to be very useful in ascites, anascara, cold, cough, fever, asthma, constipation, calculus, dropsy and enlargement of abdominal viscera. It is given only when a drastic purgative is required as in dropsy and cerebral affections like convulsions, insanity and

other fevers, attended with high blood pressure. Wood is diaphoretic in small doses and purgative and emetic in large doses.

Euphorbia hirta Linn. (Dudhi, Cara) : Cara is demulcent, antispasmodic, anti-asthmatic pectoral, anthelmintic and local parasiticide. Plant is chiefly used in the affections of childhood, in worms, bowel complaints and cough, in postnatal complaints, failure of lactation, breast pain. Extract of plant has depressant action and action on cardiovascular system, a sedative effect on mucous membranes of the respiratory and urino-genitory tract. Juice of plant is given in dysentery and colic, and milk applied to destroy warts. Plant alkaloid is effective in respiratory system and produces dilation of bronchi. Decoction of plant is used in bronchial affections and asthma. Latex is vermifuge and used in diseases of urino-genitory tract and also in application for warts.

Euphorbia tirucalli Linn. (Vajraduhu, Satsala) :It is useful in biliousness, leucorrhoea, leprosy, dropsy, whooping asthma, enlargement of spleen, dyspepsia, jaundice, colic tumours, and stones in bladder. Milky juice is vesicant and rubifacient. In small doses a purgative but in large doses it is acrid, emetic and counter-irritant; application for warts, neuralgia, rheumatism, toothache, asthma, cough and earache. It is also a fish poison. Milky juice is applied to itch and scorpion bites. Decoction of tender branches and that of roots is administered in colic and gastralgia.

Anti-HIV agents among desert plants

Around 40million people are affected due to the Human Immuno-deficiency Virus globally. During the past decades, a large number of anti-viral screening experiments on medicinal plant extracts have been reported and have led to the selection of several extracts active towards herpes viruses. A promising result of a naturally occurring antiherpetic agent was given by n-docosanol (a natural 22 carbon saturated fatty alcohol) which is undergoing phase III clinical trials in patients. Clinical testing of the topical formulation, or systemic administration of drug suspensions has demonstrated a good therapeutic index, since high doses of n-docosanol do not elicit appreciable toxicity. The findings show that natural products are still potential sources in the search for new antiherpatic agents(Hattori et al., 1995,). Various plant extracts used in Ayurvedic medicine for inhibitory effects on HIV virus have been studied (Hattori et al., 1993 ; Kusumoto et al., 1995; Hattori, personal communication). A large number of such plants occur in semi-arid and arid climate of Rajasthan (Roy and Kumar, 1995) .

Acquired immunodeficiency syndrome (AIDS) , the great pandemic of the second half of the 20th Century, is still a threatening disease world wide. Many research approaches are currently aimed at developing novel agents to arrest the replication of HIV through various targets. These may include the inhibition of reverse transcriptase (RT), protease (PR), membrane fusion and integrase. HIV PR enzyme has been demonstrated to play an essential role in viral replication (Meek et al., 1990). It is considered as potential target for anti-AIDS therapy, as the inhibition of this enzyme produces immature, non-infectious virions (Mous et al., 1988; Huff, 1991; Robins and Platter, 1993). A range of

HIV PR inhibitors have been designed and applied in clinical trials such as Sanqunavir, Ritonavir and Indinavir. However, the development of drug resistance by virus, irrespective of the target, remains as an overwhelming problem in AIDS chemotherapy (El Farrash et al. 1994). Thus there is great need to search for and develop new and different anti-HIV candidates from plants and natural products are of considerable importance.

In search for anti-HIV active agents from natural products, many attempts at screening traditional medicines have been made (Chang and Yeung, 1989; Otake et al., 1995; Wan et al., 1996). However Indian and other tropical region plants with their vast diversity, have not been investigated for their antiviral activity. Hussein et al. (1999) investigated forty eight methanol extracts from Sudanese plants which were screened for their inhibitory activity on viral replication. Nineteen extracts showed inhibitory effects on HIV–induced cytopathic effects (CPE) on MT-4 cells. The extracts were further screened against HIV-1 protease (PR) using an HPLC assay method. Of the tested extracts, the methanol extracts of the desert plants *Acacia nilotica* (bark and pods), *Euphorbia granulata* (leaves) , *Maytenus senegalensis* (stem-bark) and aqueous extracts of *A. nilotica* (pods) and *M. senegalensis* (stem-bark) showed considerable inhibitory effects against HIV-1 PR (Hussain et al., 1999) . Some of the plants from Sudan are common within the Indian dessert region of Rajasthan and generally they grow on the wastelands. They have potential use as bio-energy plantations (Kumar et al., 1995; Kumar, 1998). However a large number of them are used in the medicines of Ayurveda. They were also found effective against HIV-1. (Hussein et. al., 1999) . A list of potential plants of this region is given here in table 1. However these plants have not been studied in detail and there is need to study them for their medicinal properties including anti-HIV properties. Some of the active principles against anti-HIV are triterpenoids which are abundant in laticiferous plants of Rajasthan. Besides, *Ganoderma* sp is very frequently met in Rajasthan attacking trees. *Ganoderma lucidum* has been described to contain triterpenes which have inhibitory effects against HIV-1 protease (Min et al., 1998). Besides this, several other plants like *Abrus precatorius* L., Leguminosae (Chao-mei et al., 1998) , *Datura stramonium* L., *Balanites aegyptiaca* L. Delile etc. commonly found in Rajasthan show anti-HIV activity (Kawahata et al., 1996). In China, its seeds have been used as an insecticide and for skin diseases since ancient times .

A detailed survey of medicinally important plants has been carried out and important trees, shrubs and herbs have been listed and their characters studied in several publications from our laboratory. They included drugs for cure of urinary tract infection (Ajanta and Kumar, 2000b) anti-depressant herbal drugs (Ajanta and Kumar, 2000c), medicines for skin diseases (Shivani and Kumar, 2000), anticancer drugs (Sharma and Kumar, 2000); anti-diabetic drugs (Ritu and Kumar, 2000). Herbal drugs of Leguminosae from Rajasthan have been studied (Sapna and Kumar, 2000). Herbal crude drugs for anti-malaria (Anita and Kumar, 2001); anti-paralytic (Vandana and Kumar, 2001). Besides this, herbal crude drugs for cure of hepatic diseases (Santosh and Kumar, 2001) and diseases of the digestive system (Mamta and Kumar, 2001) have been studied for their characters and investigations on their morphological and pharmacognostical characterization are in progress.

Conclusion

The sustainable land utilization in the ecologically fragile climate of semi arid and arid regions has to be guided by the principal of optimal utilization of resources. It is a matter of great interest that a large number of plants of the arid and semi-arid regions of the world are effective as anti-HIV agents. They are also used in variety to herbal and traditional medicines as listed in this paper. Our previous work on their bio-energy production potential, if combined with their crude drug potential could yield bio-fuels on one hand and valuable crude drugs on the other. However a large number of tropical plants have not been studied in detail for their chemical constituents, pharmacological properties of the extracts, and their pharmacognostical characterization including DNA sequencing etc. If a joint collaboration could be established in this direction, valuable information could be generated with wide ranging practical applications. This could also provide alternative land use pattern for the rural poor thriving on marginal lands on one hand and help in eco-restoration on the other. The use of bio-energy plants in the herbal crude drugs has great potential and detailed investigations are planned with the help and cooperation of different agencies. This paper provided a brief outline of the work in the area for future suggestions and improvement.

Acknowldegement

I am deeply indebted to Professor Watanabe, Director of the Research Institute of Wakan Yaku for providing me an opportunity to work as visiting Professor at the Institute and Dr. Komatsu for constant encouragement support during my stay and work at the TMPU. Toyama.The keen interest and encouragement by Professor Dr. Neumann during the course of this work is gratefully acknowledged.

References

Arora, R.K. and E.R. Nayar, (1984): Wild relatives of crop plants of India. NBPGR. Science Monograph No. 9, New Delhi.

Byung-Sun, M., N. Nakamura and H. Miyashiro (1998): Triterpenes from the spores of *Ganoderma lucidum* and their inhibitory activity against HIV-protease. Chem. Pharm.Bull. 46 (10), 1607-1612.

Chang, R.S. and H.W. Young (1989): Inhibition of the growth of human immunodeficiency virus in vitro by crude extracts of Chinese medicinal herbs. Antiviral Res. 9, 163-176.

Chao-mei, M., N. Nakamura and M. Hattori (1998): Saponins and C-Glycosyl Flavones from the seeds of Abrus precatorius. Chem. Pharm. Bull. 46 (6), 982-987.

Choudhary, M. and A. Kumar (2001): Ayurvedic crude drugs for cure of diseases of the digestive system. Int. J. Mendel. 18, 27-28.

El-Farrash, M.A., M.J. Kuroda and T. Kitazaki, et. al. (1994): Generation and characterization of a human immunodeficiency virus type 1 (HIV-1) mutant to an HIV-protease inhibitor. J. Virol. 68:233-239.

Gupta R. and Kumar, A. (2000): Ayurvedic crude drugs as potential cure of diabetes. Int. J. Mendel. 17(3-4), 127-128.

Hattori, M., I.T. Kusumoto, M. Soga and T. Namba (1993): Screening of various Ayurvedic medicines for their inhibitory activities on the reverse transcriptase and Identification of arecatanins, an embelin, as major inhibitory substances from Areca catechu and Embelia ribes. J. Med. Pharm. Soc. Wakan-Yaku 10, 141-148.

Hattori, M., T. Nabakayashi and Y. Lim et al. (1995): Inhibitory effects of various Ayurvedic and Panamian medicinal plants on the infection of Herpes simplex virus 1 in vitro and in vivo. Phythother. Res. 9, 270-276.

Huff, J.R. (1991): HIV-1 protease: A novel chemotherapeutic target for AIDS. J. Med. Chem. 34, 2305-2314.

Hussein, G., H. Miyashiva, N. Nakamura, M. Hattori et al. (1999): Inhibitory effects of Sudanese plant extract on HIV-1 replication and HIV-1 Protease. Phytotherapy Res. 13, 31-36.

J.R.Goodin and D.K. Nottington (eds.). International Center for Arid and Semi-arid land studies, Lubbock, Texas, pp. 261-281.

Kawahata, ,T., T. Otake, H. Mori, M. Morimoto, N. Ueba, I.T. Kusumoto, S. El-Mekkawy, M.Hattori, T. Namba (1995): J. Trad. Med. 13, 59-65.

Kotia, A. and A. Kumar (2001b). Some of the common weeds of medicinal value from Rajasthan . Int. J. Mendel. 18, 17-20.

Kotia, A. and A. Kumar (2001a): Characterization of weeds on wastelands and their role in eco-development . Int. J. Mendel. 18, 7-8.

Kumar, A. (1998): Biomass energy crops of semi-arid regions of India and their energy potential. In: Biomass for Energy and Industry (H. Kopetz et al., eds.) CARMEN, Germany. pp 345 – 348.

Kumar, A. , S. Johari, and S. Roy (1995): Production and improvement of bioenergy sources. J. Indian Bot. Soc. 74A, 233-244.

Kumar, A. (2000): Hydrocarbon yielding plants and future prospects: Biotechnological approach. In: Plant Biotechnology (P.C. Trivedi, ed.) Panima. New Delhi. pp 194-212.

Kusumoto, T. Nakabayashi H. Kida et al. (1995): Screening of various plant extracts used in Ayurvedic medicine for inhibitory effects on human immunodeficiency virus type 1 (HIV-1) protease. Phytother. Res. 9, 180-184.

Meek, T.D., D.M. Lambert, B.W. Metcalf, S.R. Petterway and G.B. Dreyer (1990): HIV-1 protease as target for potential anti-AIDS drugs. In: Design of Anti-AIDS Drugs. E. DeClerckq,ed. Elsevier Science Publishers B.V. , The Netherlands, pp. 225-256.

Mishra, A. and A. Kumar (2001b): Studies on Ayurvedic crude drugs for the cure of urinary tract stones. Int. J. Mendel 18 (1-2), 41-42.

Mishra, A. and A. Kumar (2001a): Medicinally important trees of Rajasthan. Int. J. Mendel. 18, 37-38.

Mishra, A. and Ashwani Kumar (2001c): Searching for anti-depressant crude drugs in ayurvedic system of medicine. Int. J. Mendel. 17 (3-4), 91-92.

Mous, J., E.P. Heimer and S.F.J. Le Grice (1988): Processing protease and reverse transcriptase from human immunodeficiency virus type 1 polyprotein in Escherischia coli. J. Virol. 62, 1433-1444.

Otake, T., H. Mori, M. Morimoto et al. (1995): Screening of Indonesian plant extracts for anti-human immunodeficiency virus type 1 (HIV-1) activity. Phytother. Res. 9, 6-10 .

Paroda, R.S. (1979): Plant resources of Indian arid zones for industrial uses. In: Arid land Plant resources.

Robbins, T. and J. Platter, (1993): HIV protease inhibitors, their anti-HIV activity and potential role in treatment. J. AIDS. 6, 162-170..

Roy, S. and A. Kumar (1995): Biodiversity of Rajasthan and its energy potentials. J. Enviroment and pollution. 2(3), 105-109.

Sanghi, S. and A. Kumar (2000): Characterization of some of the medicinal plants of family Leguminosae used in Ayurvedic system of medicines. Int. J. Mendel. 17, 109-110.

Sharma, L.K. and A. Kumar (2000): Searching for anti-cancer drugs in traditional medicines. Int. J. Mendel. 17, 77-78.

Sharma, S. and A. Kumar (2001): Ayurvedic plants for cure of hepatic diseases. Int. J. Mendel. 18: 13-14.

Sharma, V. and A. Kumar (2001): Studies on anti-paralytic crude drugs used in Ayurvedic system. Int. J. Mendel. 18, 33-34.

Shivani and A. Kumar (2000): Ayurvedic medicinal plants used for skin diseases. Int. J. Mendel. 17, 105-106.

Wan , M., S. Bloor, L. Foo and B. Loh (1996): Screening of New Zealand plant extracts for inhibitory activity against HIV-1 protease. Phytother. Res. 10, 589-595.

Yadav, A. and A. Kumar (2001): Studies on anti-malarial drugs used in Ayurvedic system. Int J. Mendel Int. J. Mendel. 18(1-2), 29-30.

The Influence of Calcium and Low Temperatures on Oryzalin-induced Reactions of Wheat Roots – Physiological and Biochemical Aspects.[+)]

Elena Asafova*[)], Olga Timofeeva, Olga Olinevich and Ludmilla Khokhlova

State University of Kasan, Faculty of Biology, Department of Plant Physiology and Biotechnology

*[)] Corresponding author; e-mail: Elena.Asafova@ksu.ru

[+)] Translated by L. Bender

Abstract

It was shown that the inhibitor of tubulin protein polymerization, oryzalin (10 μM) evoked structural changes in the principal part of microtubules in cells of wheat seedling roots and brought about an increase in electrolyte exosmosis from the root tissue as well as an increase in cell wall lectin activity while soluble lectin activity and soluble protein content were decreased. After cold hardening of the seedlings at +3 °C for 7 days an increase of the spatial microtubule aggregation and a decrease of the oryzalin effect were noted. Exogenous $CaCl_2$ (1mM) and the antagonist of the Ca^{2+}-calmodulin complex, chlorpromazin (250 μM) influenced, possibly because of changes in the phosphorylation of proteins interacting with the microtubules and the membraneotrope action of these substances.
It is assumed that the change of cytoskeleton stability brought about by the influence of the external stimuli is linked to the functioning of the cell signaling system, thus defining the biochemical and the physiological reactions of the plants.

Introduction

Calcium is one of the most important regulator ions in cell metabolism. Supposedly, Ca^{2+} ions may act as a link between „soluble“ signals and the cytoskeleton configuration (Fulton,1987). It is known that the maintainance of the cytoskeleton structure and its function depend on the Ca^{2+} concentration in the cytosol (Schliwa et al., 1981; Williamson, 1987).

Microtubules are reported to be stable if the Ca^{2+} concentration in the cells is below 1 µM while mM concentrations initiate rapid destruction of the tubulin proteins (Schliwa et al., 1981). The changes of the structural and polymerization state of the cytoskeleton filaments are brought about by association proteins which act through the activation of the Ca^{2+} - calmodulin complex, i.e. phosphorylation processes (Cyr, 1991).
Recent models suggest that cells contain a uniform system of functional structures which links plasmalemma and cortical microtubules (Sonobe and Takahashi, 1994) as well as lectins and the cytoskeleton (Timofeeva et al., 1999). It is assumed that the stability of cortical microtubules increases if they interact with transmembrane proteins (Loyd et al., 1996). These components ensure signal transduction within the cell where these signals will be transmitted to cellular lectins and proteins. It is now acknowledged that the cytoskeleton is an important factor within the signal transmitting systems of plant cells (Nick, 1998), where the linkage of microtubules to the plasmalemma possibly acts as the target for the cellular signaling system. Traditionally, specific microtubule and microfilament inhibitors are used in studies on the role of the cytoskeleton in cellular processes. In the investigations reported here we used the highly specific inhibitor of the polymerization of plant tubulins – oryzalin which belongs to the chemical group of dinitroaniline herbicides (Morejohn et al., 1987). Still, it is not known whether this inhibitor acts through direct influences on the cytoskeleton structure or via the regulation of cytosolic calcium. The aim of our investigations was to study the permeability of the plasmalemma, the activity of lectins and the content of soluble protein in connection with structural modifications of the cytoskeleton and the Ca^{2+}-calmodulin system under the influence of oryzalin on plants at optimal and suboptimal temperatures.

Material and methods

The studies were carried out using roots of wheat seedlings (Triticum aestivum L., cultivar Mironovskaya 808). The seedlings were incubated for 7 days in either bidistilled water or 1 M $CaCl_2$ at 23 ± 2 °C and a 12 hour photoperiod (100 watts m^{-2}). Cold treatments were also performed for 7 days at 3 ± 1 °C. The antagonist of the Ca^{2+}-calmodulin dependend reaction, Chlorpromazin (0.25 mM) was applied to the medium 24 hours before the cold treatment commenced. Excised roots were incubated in 0.01 mM oryzalin for 3 hours.

The visualization of microtubules in cells of the differentiation zone (6 – 7 mm behind the root tip) was carried out employing indirect immunofluorescence microscopy according to Baluska et al. (1997) using Stidman wax (low melting point) and primary monoclonal α-tubulin antibodies (Amersham, N 356; Sweden) as well as secondary antibodies – anti-mouse immunoglobulin conjugated with biotin (Amersham, RON 1001). Streptawidin-fluorescin (Amersham, RPN 1232) was used as a fluorescent dye. 25 preparations per treatment were investigated employing the fluorescence microscopy system Leica DMLB (Germany) using the software Image-Pro-Plus (DELL Optiplex GX1p). Stability of microtubules was expressed as the percentage of preparations showing polymerized microtubules after the incubation with oryzalin.

The permeability of the plasmalemma was determined conductometrically after incubating wheat roots in distilled water (25 mg root material per 25 ml of water for 2 hours at constant agitation with a magnetic stirrer). Electrical conductivity of the incubation solution was expressed as a percentage of maximum electrolyte exosmosis obtained with root tissue incubated at 100 °C for 30 minutes.

Soluble lectins were extracted with 0.05 M HCl (tissue : medium ratio 1 : 10) for 1 hour. After centrifugation, 1 M Na-phosphate buffer pH 7.2 – 7.4 was added to the supernatant (extract : buffer ratio 2 : 1). This solution was used for the determination of soluble lectin activity. The cell wall bound lectins were recovered from the remaining pellet reextracting it for 4 hours with a medium consisting of one volume of 0.2 M sucrose + 0.02 M KH_2PO_4 + 0.01 M EDTA and six volumes of 0.05 % Triton-X + 0.9 % NaCl (pH 5.0). Here, tissue : medium ratio was 1 : 2. Lectin activity in the supernatants was assayed by the agglutination of trypsin treated erythrocyte preparations and determined as the minimum protein concentration yielded through agglutination. The concentration of soluble proteins and total protein content were measured following the methods of Kumar et al. (1983) and Bradford (1976), respectively. All experiments were carried out in triplicate and the results treated statistically.

Results

In roots of wheat seedlings oryzalin increased the permeability of the plasmalemma by 11 % (Figure 1A) and the activity of the cell wall lectins by 45 % (Figure 2A) at optimal temperatures. These results reflect the changes of microtuble structure in the root cells. Figure 3A shows the structural changes of native microtubules under the influence of this inhibitor which reduces the preparations exhibiting polymerized tubulin structures to 5 % of that found

in the control treatments (Table 1). It could also be demonstrated that the activity of the soluble lectins decreased by about 70 % (Figure 4A) and the content of soluble protein by 88 % (Figure 5A).

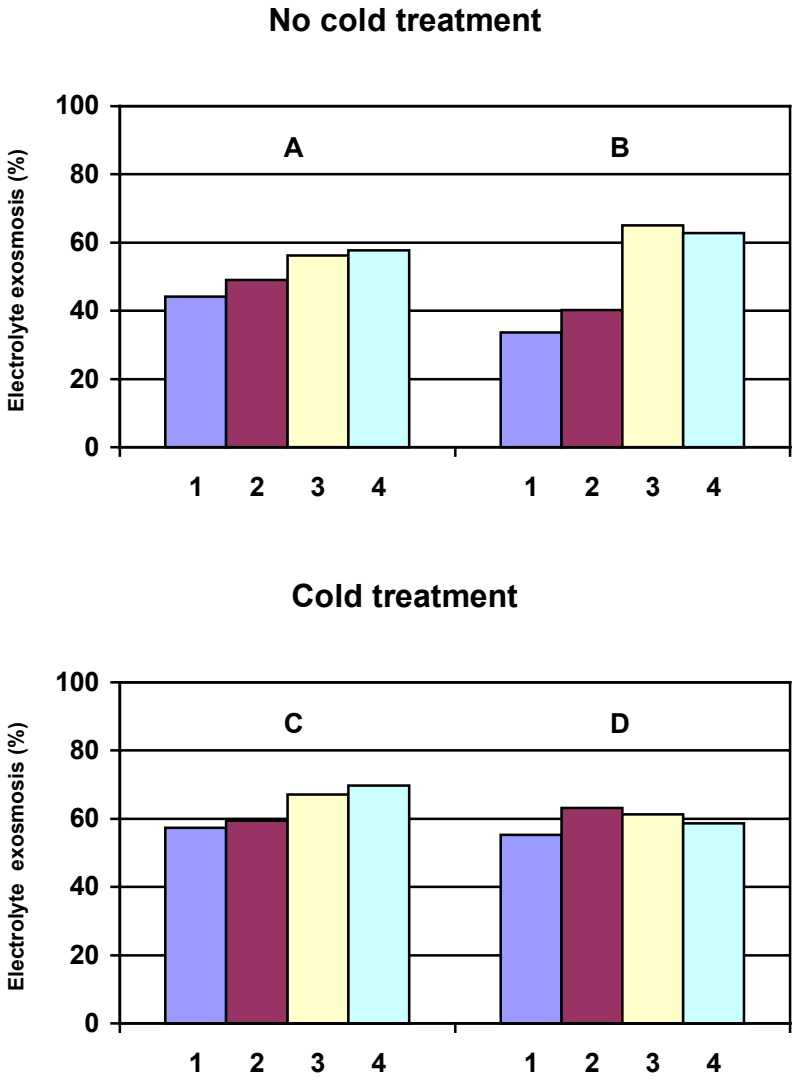


Figure 1: Plasmalemma permeability in roots of wheat seedlings as influenced by oryzalin and chlorpromazin: A, C, without Ca^{2+}; B, D, with Ca2+; 1, control; 2, oryzalin (10 µM); 3, chlorpromazin (250 µM); 4, oryzalin + chlorpromazin.

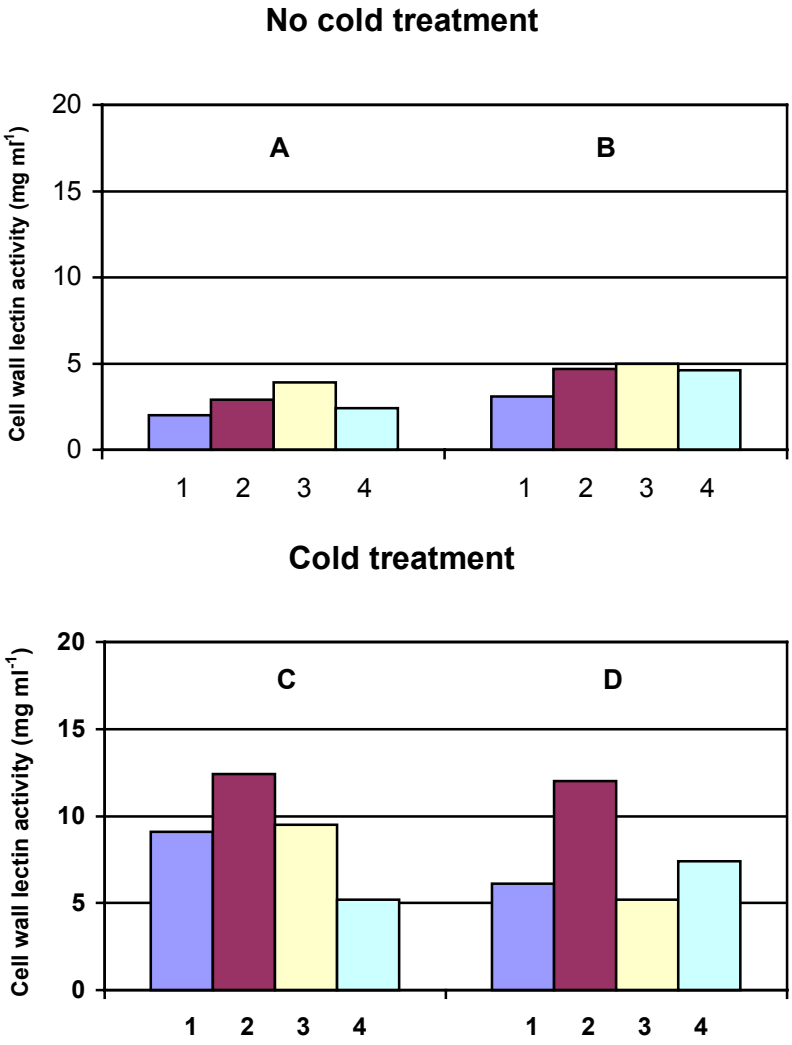


Figure 2: Cell wall lectin activity in roots of wheat seedlings as influenced by oryzalin and chlorpromazin: A, C, - Ca^{2+}; B, D, + Ca^{2+}; 1, control; 2, + oryzalin (10 µM); 3, + chlorpromazin (250 µM); 4, + oryzalin + chlorpromazin.

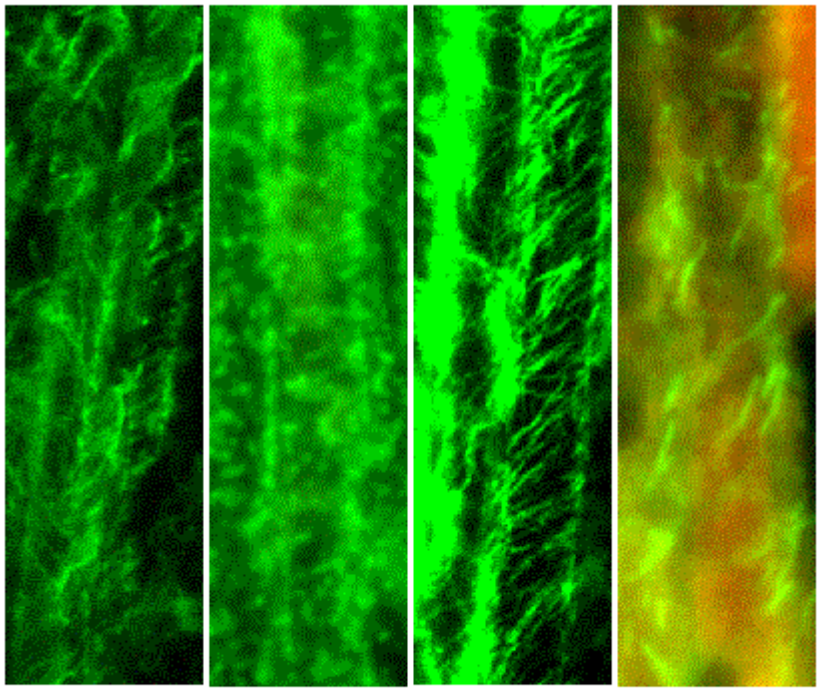


Figure 3: The visualization of the microtubule cytoskeleton in cells of the differentiation zone of wheat seedling roots. A, control (23 °C); B, + oryzalin (10 μM) at 23 °C; C, cold treatment (3 °C, 7 days); D, cold treatment + oryzalin (10 μM).

Table 1: The influence of oryzalin on the percentage of polymerized microtubule at high and low temperatures.

	23 ± 2 °C	3 ± 1 °C
- Oryzalin	100	100
+ Oryzalin	5	28

Chlorpromazin caused a 27 % increase of the electrolyte exosmosis from the root tissue (Figure 1A) and the activity of cell wall lectins had almost doubled (Figure 2A) while the activity of the soluble lectins (Figure 4A) and the soluble protein (Figure 5A) decreased by 77 and 38 %, respectively. Similar changes could be demonstrated in media with and without Ca^{2+} under the influence of oryzalin which increased the activity of cell wall lectins (Figure 2B) whereas the activity of soluble lectins was decreased (Figure 4B).

No differences in the influence of chlorpromazin on lectin activity could be found in media with and without Ca^{2+} while higher electrolyte exosmosis from the tissue and a more pronounced decrease in soluble protein content were observed in the Ca^{2+} treatment.

Combined application of chlorpromazin and oryzalin did not result in additive effects. In this case all tested parameters yielded similar data as with chlorpromazin (Figures 1A and B, 2A and B, 4A and B, 5A and B).

Oryzalin did not influence electrolyte exosmosis after cold adaptation (Figure 1C) while under this condition microtubule aggregation was more pronounced (Figure 3C and D).

In the cold treatments oryzalin reduced the number of preparations exhibiting polymerized microtubules to a considerably lesser degree than at 23 °C (Table 1) whereas the other parameters responded as at 23 °C. The cold adaptation caused a smaller change of plasmalemma permeability (Figure 1C) and the activity of cell wall lectins (Figure 2C) under the influence of chlorpromazin whereas the reduction of the soluble lectin activity (Figure 4C) and the soluble protein content (Figure 5C) was considerably stronger.

After the application of exogenous calcium an increase in electrolyte exosmosis of 14 % (Figure 1D) and a doubling of cell wall lectin activity (Figure 2D) was noted in the oryzalin treatment while calcium did not affect the influence of oryzalin on the content of soluble protein (Figure 5C and D). The effect of chlorpromazin on the permeability of the plasmalemma (Figure 1D) and the activity of cell wall lectins (Figure 2D) was reduced in cold adapted (hardened) roots in both the plus and minus calcium treatment while the reduction of the soluble lectin activity (Figure 4D) and the soluble protein content (Figure 5D) due to the chlorpromazin treatment were the same at 23 and 3 °C. In cold adapted roots no additional effects on the tested parameters could be seen if chlorpromazin and oryzalin were applied in combination.

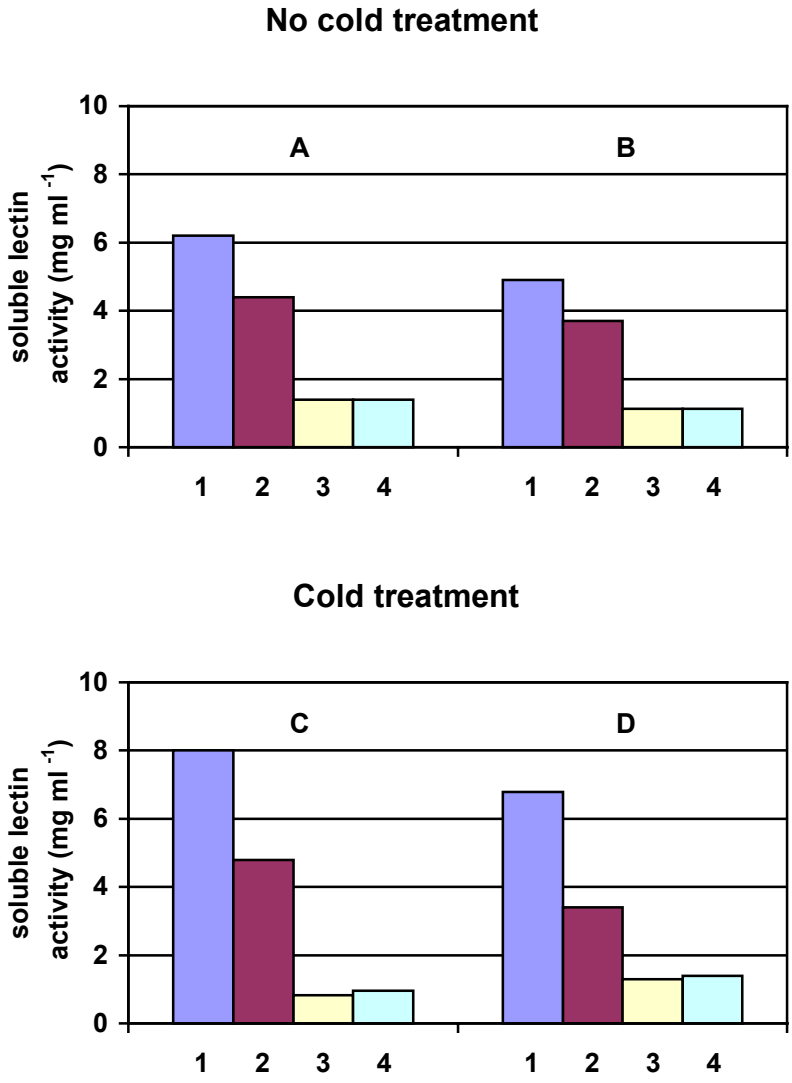


Figure 4: Soluble lectin activity in roots of wheat seedlings as influenced by oryzalin and chlorpromazin: A, C, - Ca^{2+}; B, D, + Ca^{2+}; 1, control; 2, + oryzalin (10 µM); 3, + chlorpromazin (250 µM); 4, + oryzalin + chlorpromazin.

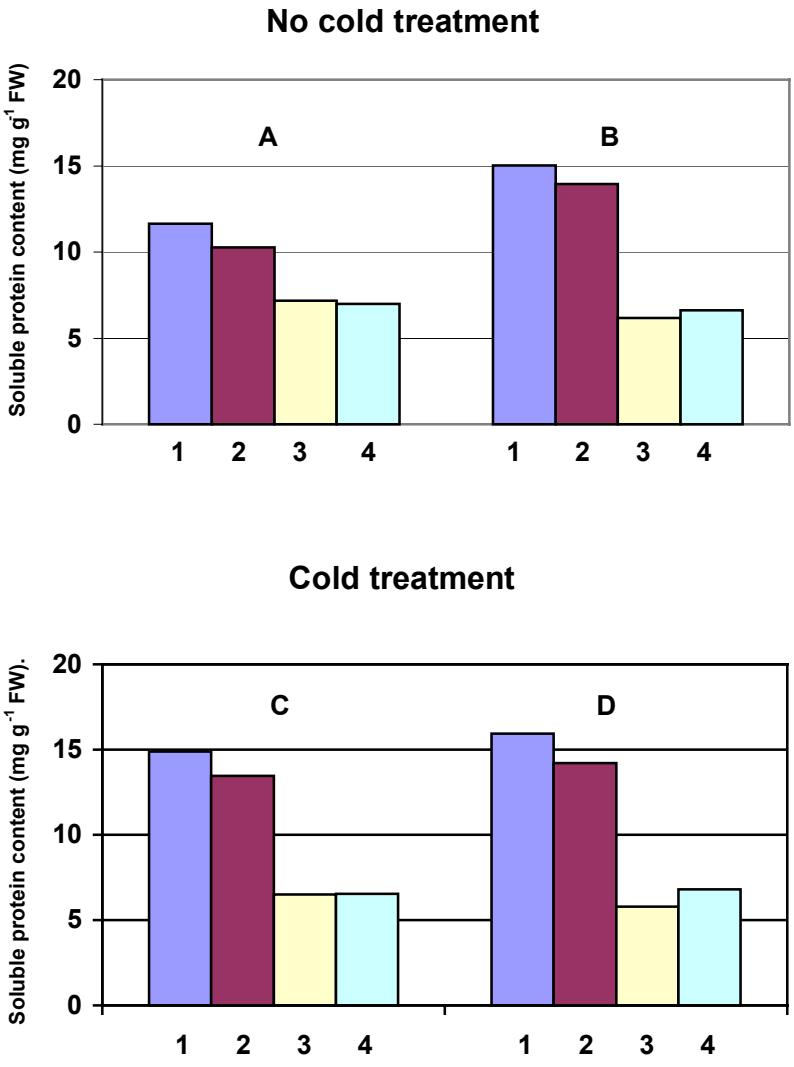


Figure 5: Soluble protein level in roots of wheat seedlings as influenced by oryzalin and chlorpromazin: A, C, - Ca^{2+}; B, D, + Ca^{2+}; 1, control; 2, + oryzalin (10 μM); 3, + chlorpromazin (250 μM); 4, + oryzalin + chlorpromazin.

Discussion

Our data show that at optimal temperatures (23 °C) the structure of microtubules was altered under the influence of oryzalin. Binding to tubulin, oryzalin inhibits the polymerization, fosters the disintegration of existing and impedes the formation of new microtubules (Morejohn et al., 1988) – perhaps because of the reduction of the tubulin content (Giani et al., 1998). Apparrently, the microtubule disintegration observed in our studies enhanceded the changes in cell reactions, i.e. the increase in plasmalemma permeability and cell wall lectin activity as well as the reduction of soluble lectin activity and soluble protein content.

On the other hand, cold adaptation enhanced the structural stability of the tubulin cytoskeleton thereby counteracting the effect of oryzalin. This can probably be related to the synthesis of new microtubule association proteins (MAPs; Cyr and Palevitz, 1989) and the improvement of the interaction of cortical microtubules with the plasmalemma (Baluska et al., 1993) which resulted in the formation of cold stable membrane-cytoskeleton complexes and a reduction of plasmalemma permeability. Hence, the electrolyte exosmosis observed under the influence of oryzalin is reduced in cold adapted (hardened) roots of wheat seedlings. In an earlier report, we demonstrated the maintenance of free Ca^{2+} in oryzalin treated seedling leaves at optimal temperatures (Asafova et al., 1999). In the investigations described here the same effect of oryzalin on the permeability of the plasmalemma and the activity of cell wall bound and soluble lectins in seedling roots incubated with and without Ca^{2+} at optimal temperature conditions could be demonstrated. This was the basis for the assumption that exogenous Ca^{2+} had no influence on cytosolic Ca^{2+} in root cells and that the disorganization of the tubulin cytoskeleton was not enhanced by an increase of free Ca^{2+}. Keifer et al. (1992) deny the influence of μM concentrations of oryzalin on the Ca^{2+} status of the cell. Yet, there are other results: Oryzalin increased the activity of Ca^{2+} channels (Thion et al., 1996) and in our experiments the reduction of the soluble protein content due to the oryzalin treatment was the same with and without Ca^{2+} amendment. It is also known that protease activities are under the control of Ca^{2+} ions (Tarchevsky, 1993) which supportes our assumption as to the maintenance of the Ca^{2+} status of the cells. This interpretation is in accordance with the results of Sonobe (1990) who could not find disorganisation of microtubules in a medium containing 1 mM $CaCl_2$. As was outlined earlier, the disintegration of microtubules is related to the increase of Ca^{2+} in the cells (Schliwa et al., 1981). Cyr (1991) was able to demonstrate that 1 mM Ca^{2+} concentration in the cytosol of carrot protoplasts destabilized their microtubules.

The increased oryzalin sensitivity of the root cell plasmalemma induced by cold adaptation and Ca^{2+} can be interpreted as the consequence of the time lag for the formation cold resistant microtubule poulations. Further, changes in the phosphorylation status of proteins which are linked to the microtubules or a destabilizing effect of the Ca^{2+}-calmodulin complex on cortical microtubules may have caused the observed effects (Fisher et al., 1996).

In order to test this assumption we used chlororomazin which inhibits the formation of the Ca^{2+}-calmodulin complex. Under optimal temperature conditions this inhibitor diminished the activty of soluble lectins and, simultaneously, increased that of cell wall bound lectins in roots of seedlings grown with and without Ca^{2+}. According to modern views calmodulin stabilizes cortical microtubules (Fisher et al., 1996). Perhaps, chlorpromazin is more effective in microtubule disorganization than oryzalin. This may explain the observation that no effect of oryzalin could be seen in chlorpromazin treated tissue. Chlorpromazin does not only influence the organization of microtubule due to the destruction of the Ca^{2+}-calmodulin complex but may also function as a membraneotropic agent. This is confirmed by our results which showed that electrolyte exosmosis was increased to a greater extent in chlorpromazin treated roots than under the influence of oryzalin and that applied Ca^{2+} enhanced this effect.

In the literature it was discussed that one reason for the destabilizing effect of the Ca^{2+}-calmodulin complex is its capability to destroy the contacts between microtubules and the plasma membrane (Fisher et al., 1996). Cold treatment diminished some of the chlorpromazin effects in both the plus and minus Ca^{2+} treatments. These phenomena may be linked to the formation of Ca^{2+}-resistant microtubule populations as well as to the capability of exogenous Ca^{2+} to improve cold hardening. This could have been brought about through the aggregation of membrane components by Ca^{2+} and the improvement of plasmalemma elasticity due to the formation of Ca^{2+}-bridges between phospholipids and proteins (Issabekov and Krassavcev, 1989).

It is assumed that the changes of the stability of the tubulin cytoskeleton and its interactions with the plasmalemma is one of the links in the chain of signaling systems. These systems are regulated by exogenous calcium and low temperature which govern the biochemical and physiological responses of the plant. Therefore, it can be concluded that the cytoskeleton functions as a mediator between exogenous signals and cell reactions in plants.

References

Asafova, E., L. Khokhlova, I. Volovnik, R. Chestnova, E. Makarova, K.-H. Neumann and Ch. Lein (1999): Der Einfluss des Oryzalin, eines Inhibitors der Tubulinpolymerization bei unterschiedlichem Calcium- und Temperaturniveau bei Winterweizenkeimpflanzen. In: Die Vielfalt der Zusammenarbeit. Zum zehnjährigen Jubiläum des Vertrags über die Zusammenarbeit zwischen der Staatlichen Universität Kasan und der Justus-Liebig-Universität Giessen. Kasan Unipress, pp 366-377.

Baluska, F., J.S. Parker, P.W. Barlow (1993): The microtubular cytoskeleton in cells of cold-treated roots of maize (Zea mays L.) shows tissue-specific responses. Protoplasma 172, 84-96.

Baluska, F., S. Vitha, P.W. Barlow and D. Volkmann (1997): Rearrangements of F-actin arrays in growing cells of intact maize root apex tissues: A major developmental switch occurs in the postmitotic transition region. Europ.J.of Cell Biol. 72, 113-121.

Cyr, R.J. (1991): Calcium/calmodulin affects microtubule stability in lysed protoplasts. J. Cell Sci. 100, 311-317.

Cyr, R.J. and B.A. Palevitz (1989): Microtubule-binding proteins from carrot. 1. Initial characterization and microtubule bundling. Planta 177, 245-260.

Fisher, D.D., S. Gilroy and R.J. Cyr (1996): Evidence for opposing effects of calmodulin on cortical microtubules. Plant Physiol. 112, 1079-1087.

Fulton A. (1987): Cytoskelett. Architektur und Choreographie. Mir publishers, Moscow , 117 pages.

Giani, S., X. Qin, F. Faora, D.Brevirio (1998): Oryzalin and protein levels in rice. Planta 205, 334-341.

Issabekov, B.N. and O.A. Krassavcev(1989): Osmotische Eigenschaften der frostresistenten Protoplasten. Russ.J.of Plant Physiol. 36/2, 372-386.

Keifer, A.Q., D.A. Callacham and P. Hepler (1992): Inhibitors of cell division and protoplasmic streaming fail to cause a defeatable effect on intracellular calcium levels in stamen-hair cells of Tradescantia virginiana L. Planta 186, 361-366.

Kumar, A., L. Bender, B. Pauler, K.-H. Neumann, H. Senger and C. Jeske (1983): Ultrastructural and biochemical development of the photosynthetic apparatus during callus induction in carrot root explants. Plant Cell Tiss. Org. Cult. 2, 161-177.

Lloyd, C.W., B.K. Drobak, S.K. Dova and C.J. Staiger (1996): Interaction between the plasmamembrane and the cytoskeleton in plants. In: M. Smallwood, J.P. Knpx, D.J. Bowles (eds.) Membranes: Spezialized Functions in Plants. BIOS Scientific Publishers(UK) pp 1-20.

Morejohn, L.C., T.E. Bureau, J. Mole-Bajer, A.S. Bajer and D.E. Fosket (1987): Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. Planta 172, 252-264.

Nick, P. (1998): Signaling to the microtubular cytoskeleton in plants. Intern. Review of Cytology 184, 33-79.

Schliwa, M., U. Euteneuer, J. Ch. Bulinski and J.G. Izant (1981): Calcium lability of cytoplasmic microtubules and its modulation by microtubule-associated proteins. Cell Biology 78/2, 1037-1041.

Sonobe, S. (1990): ATP-dependent depolimerization of cortical microtubules by an extract in tobacco BY-2 cells. Plant Cell Physiol. 31, 1147-1153.

Sonobe, S. and S. Takahashi (1994): Association of microtubules with the plasma membrane of tobacco BY-2 cells in vitro. Plant Cell Physiol. 35/3, 451-460.

Tarchevsky, I.A. (1993): Katabolism und Stress bei Pflanzen. Nauka publishers, Moscow, 80 pages.

Thion, L., C. Mazars, P. Thuleau, A. Graziana, M. Rossignol, M. Moreau and R. Ranjeva (1996): Activation of plasma membrane voltage-dependent calcium-permeable channels by disruption of microtubules in carrot cells. FEBS Letters 393, 13-18.

Timofeeva, O.A., L.P. Khokhlova, T.V. Trifiniva, N.E. Belyaeva, J.J. Chulkova (1999): Effect of Cytoskeleton Modifiert on the Lectin Activity during Plant Acclimation to low Temperatures and under ABA Treatment. Russian J. of Plant Physiology 46/2, 147-151.

Vasiljev, A.E. (1996): Vergleichende struktur-funktionelle Charakteristik des Cytoskeletts von Tieren und höheren Pflanzen. J. Allgemeine Biologie 57/3, 293-325.

Williamson, R.E. (1987): Calcium and the plant cytoskeleton. Plant Cell Environ. 7, 431-440.

Ayurvedic Medicines: Some Potential Plants for Medicine from India

Ashwani Kumar

Energy Plantation Demonstration project and Biotechnology Center,

Department of Botany, University of Rajasthan, Jaipur, 302004 (Rajasthan) India.

e-mail: msku4@hotmail.com

Abstract

With the changing pattern of life style most of the diseases are now becoming lifestyle diseases. The traditional systems of medicine based on ancient cultures are primarily concerned with building the body strength which can help in healing the ailments and these systems rely largely on the nature cure. The Ayurvedic system has described a large number of such medicines based on plants or plant product and the determination of their morphological and pharmacological or pharmacognostical characters can provide a better understanding of their active principles and mode of action.

Introduction

Contribution of the traditional medicine to human health in the 21st Century is of paramount importance. A meeting of the International Forum on Traditional Medicine held recently (1999) at theToyama Medical and Pharmaceutical University, Toyama, Japan, reviewed the potential of traditional medicines. WHO acting director Xhang emphasized that with the changing pattern of life style most of the diseases are now becoming life style diseases. Natural medicines improve the inner strength of the body. The use of traditional medical systems has attracted so much attention that an International Health Center has been opened in July in the Toyama prefecture (Province)

Some of the oldest traditional medical systems include Chinese, Ayurvedic, Unani, Japanese and recently added homeopathy and chiropathy that is also around 200 years old. The use of traditional medicine includes (i) medication by use of medicinal plant, minerals, animal material and (ii) non medication: acupuncture and yoga.

Complementary medication includes acupuncture, herbal treatment, manual, spiritual and dietary treatments.

Toyama hospital utilizes vast amount of Chinese, Japanese and Ayurvedic medicine. Detailed studies in the areas of pharmacognosy and pharmacology are under progress (Annual report, TMPU, Toyama). Besides this the Research Center for Ethnomedicines with its Museum of Materia Medica is one of its own kind in the world under Professor Watanabe. Dr. Komatsu provides a wealth of information for all scientists engaged in the field all over the world. This includes identification, molecular characterization at DNA sequence level, chemical characterization, biotransformations and studies on effects on microorganisms to direct application in the hospital. To give an entire description will be attempted in another paper. Here a brief attempt is made to identify common goals of research in India and Japan, with an objective to attract attention of workers to the great potential that the vast bio-diversity of the Indian subcontinent and the wealth of Ayurvedic literature has to offer for future development of traditional medicines. However detailed future investigations are needed in this area to exploit the unexplored or poorly explored plant materials.

These traditional medicines have found practical application at clinical level in TMPU and over hundred cases of fissure have been cured in the hospital using a special thread prepared from latex of Euphorbia spp., thor of India (Euphorbia sp) , haldi powder (*Curcuma longa)* and some herbal ingredients. The *Euphorbia* sp is a plant of the desertic region of India and different parts of the world. A large number of energy yielding desertic plants of India used in the Ayurvedic system have great potential as Ayurvedic medicine . Negative environmental effects of current agricultural practices, such as emission of greenhouse gases, nutrient leaching, decreased soil fertility, and erosion, may be reduced when traditional annual food crops are replaced by dedicated perennial energy crops and medicinal plants. As they are able to grow and produce valuable products under dessert conditions they have great potential for covering the global desert areas into green belts leading to environmental improvement on one hand and providing valuable Ayurvedic crude drugs in addition to supplementing the bio-energy resources as renewable fuels. However detailed studies on their pharmacognostical characterization and determination of chemical products obtained from them are lacking. Some of the investigations indicated their potential use in Human immuno deficiency (HIV) diseases (Hattori et al., 1995). Such bio-energy plants have not been explored in depth. Here an attempt shall be made to provide a brief outlook of the Indian scene and highlight some of the work being carried out at our place in Rajasthan along with the possible impact assessment for desertic plants for future research strategies.
Among the desert plants the value of *Aloe vera* (L.) was recognized more than 3000 years ago when the Egyptian and Greek civilizations used its extract for skin burns, cuts and wounds on the skin surface and found that it had a wonderful healing effects on the skin. It is claimed that even 3rd degree burns can be cured and healed by *Aloe vera*. The chemical compounds like Aloein, resins and a mixture of polysaccharides containing pectic acid are present. Modern investigations indicate that extracts of *Aloe vera* act on the dead epithelial cells of the skin, aiding their removal from the surface and stimulating

the growth of new cells. Thus Aloe is a great gift of traditional medicine for protecting the smooth skin of human beings especially when radiation damage has assumed an alarming situation due to stratospheric ozone depletion. Fresh juice of leaves are also used in liver and spleen troubles and also for eye troubles, found useful in X-ray burns, dermatitis, coetaneous and other skin disorders.

In India, Egypt and Sudan around 70 percent of the rural people use traditional medicine. Similar situation exists in a large number of developing countries. In India and China 60 percent of the people affected with cholera and malaria are treated with herbal medicines. In these countries the market for traditional medicines is US $ 500 million while Western type medicine account for only 300 million US $. In Singapore 50 percent and in Australia 60 percent of population uses alternative medicine. Around 17,000 herbal products are registered in these countries. In Belgium 40 percent contemporary but 84 percent home medicines and 74 percent acupuncture medicine is utilized. In France 50 percent of the people take advantage of complementary medicine. In Germany 10,000 to 13, 000 alternative medical practitioners are thriving well and 75 percent of them utilize complementary medicines. 77 percent of pain clinics utilize acupuncture. In UK 90 percent of the complementary medical practitioners utilize osteopathy and acupuncture. In US where in 1990 only 30 percent of the people were utilizing complementary medicines, it grew to 40 percent in 1997.

Ayurvedic system of Medicine

Ayurveda is an offshoot of Atharva veda written over 3000 thousand years ago. The Charak and Sushruta describe a large number of crude drugs and a large part of them has origin to plants. However though some part of it has been translated from Sanskrit to Japanese and the Japan Society of Ayurveda under Professor Dr. Namba is very active in this field. But many of the crude drugs described remain to be identified to its plant source in botanical terms and the Institute of Traditional Medicine is the prime center for understanding the nature and morphology of crude drugs of Ayurvedic origin and their identification to the plant level. The personal communication with Professor Watanabe and Dr. Komatsu during my stay at Wakan Yaku as visiting Professor has contributed to the stimulation of such studies back home and some of the important findings are presented here. During my stay here I have worked on Nepalese crude drugs with support and guidance from Dr. Komatsu and other members of this institute.

The basic philosophy of Ayurveda considers that man is an inseparable part of the universe. The human body, mind and spirit continuum is an integral whole and the individual is also linked to the family, society, environment and ultimately the universe. The definition of health is that " It is state of complete psychosomatic equilibrium. It does not mean only absence of diseases but a state in which the mind, senses and spirit are pleasant and active". That agrees with the definition of WHO "Health is a state of complete physical, mental and social well being and not merely the absence of disease or infirmity"

India with its varied climate, soils and agro-ecology possesses an immense plant diversity, with over 15,000 species of higher plants. Both our Indian civilization as well as our diverse tribal heritage have gone a long way in conserving the wild weedy species, native land races and primitive cultivars (Fig. 1). The Indian gene center is endowed with rich flora, especially with regard to several less known yet economically important plants, ca. 160 cultivar species of economic plants, plus 56 species of lesser known cultivated food plants. Further there are ca. 320 species of wild and weedy economic types (Paroda, 1979; Arora and Nayar, 1984 ; Kumar, 1998).

The unutilized and underutilized resources

Out of 2,50,000 plant species only 10,000 or so have been exploited during the course of human civilization. A large number of hydrocarbon yielding plants are able to grow under semi arid and arid conditions and they also produce valuable hydrocarbons (up to 30 percent of dry matter) which could be converted into petroleum-like substances and used as fossil fuel substitute. They are rich in triterpenoids which are constituent to important drugs against HIV.

The potential plants

Certain potential plants were selected and attempts were made to develop agro-technology for their large scale cultivation (Kumar et al, 1995, Kumar, 1998, Kumar, 2000). A 50 ha bio-energy and medicinal plants cultivation demonstration center has been established on the campus of the University of Rajasthan to conduct the experiments on large scale cultivation of selected plants with the objective of developing optimal conditions for their growth and productivity, besides conserving the bio-diversity. Plantation of laticiferous plants and desert plants can be carried out, it could also lead to reclamation of marginal land that has already been abandoned in developed as well as developing countries. India alone has over 144 million hectare of marginal land which is about half of the total geographical area of the country. Touched only marginally by the green revolution, Africa suffers not only a dramatic nutritional problem but also an equally serious and inter linked problem of energy. Increasing scarcity of fuelwood, desertification, lack of water, food and medicines, excessive urbanization are all closely interdependent and rich biodiversity in developing countries has remained unutilized and underutilized for want of proper investigations.

There are surely opportunities for biomass of the medicinal plants in the south as well as in the north in wet climates and in dry ones but they will respond to very different schemes and strategies. There is not going to be a single unique recipe, rather multiplicity of solutions depending on climate, soil, availability of land, traditions as well as social and economic conditions. Technological improvements should lower production costs but they are unlikely to obtain significantly higher yields, as chemical and energy inputs must be reduced. The transformation of biomass into useful energy products and medicinal

compounds may however involve onsite industrial operations that could absorb at least part of the surplus man power.

As far as research is concerned we are all aware of the important progress being made in agricultural biotechnology. Genetic engineering for example is increasingly applied to crop plants for improving resistance to pests and diseases and for providing more favorable crop composition. There is a whole universe of possibilities in the use of advanced biotechnology to improve plants and processes. The natural medicine from plants has enormous possibilities for new and more effective means for curing the modern day ailments.

Natural resource

Total land area of Rajasthan is 3,42,239 sq km out of which 45.25 percent is characterized as wasteland. Large portions of this land were productive at a given time and due to man made deforestation, cattle pressure, water and wind based soil erosion, improper water management, they have turned out to be wastelands. (Kotia and Kumar, 2001a). A detailed survey on the weeds on wastelands yielded valuable data about the first colonizers. Out of the total weeds around 50 having important medicinal values while others produce related compounds. These regions are rich in bio-diversity and weeds were collected from different regions of the developing wastelands. (Kotia and Kumar, 2001b) .
Some of the medicinally important plants of Rajasthan are listed by Ajanta and Kumar, (2001a) They include species listed in table 1:

Table1: List of Medicinal plants of Rajasthan.

Plant species:	Local name
1. *Asparagus racemosus*	Satavari
2. *Chlorophytum arundinaceum*	Safed musli
3. *Curculigo orchioides*	Kali Musali
4. *Solanum surattense*	Kantkari
5. *Boerhaavia diffusa*	Santhi,
6. *Hamidesmus indicus*	Anantmool
7. *Sida cordifolia*	Bala
8. *Holarrhena antidysenterica*	Indrajo
9. *Curcuma aromatica*	Vanhaldi
10. *Oroxylum indicum*	Shyonaka
11. *Balanites aegyptiaca*	Hingot
12. *Withania somnifera*	Ashwagandha
13. *Aegle marmelos*	Bael
14. *Cassia fistula*	Amaltas
15. *Gymnema sysvestre*	Gudmar
16. *Terminalia arjuna*	Arjuna
17. *Butea monosperma*	Palas

18. *Soymida febrifuga*	Rohan
19. *Woodfordia fruticosa*	Dhavri
20. *Tribulus terrestris*	Gokhru
21. *Pedalium murex*	Badagokhru
22. *Vitex negundo*	Negad
23. *Dyerophytum indicum*	Chhitral
24. *Plumbago zeylanicum*	Chitrak
25. *Plantago ovata*	Isabgol
26. *Colocynthes vulgaris*	Indrayan
27. *Adhathoda vasica*	Ardusta
28. *Allangium salvifolium*	Aankol
29. *Caesalpinnia bonducella*	Tas
30. *Jatropha curcas*	Ratanjot
31. *Eclipta alba*	Bhringraj
32. *Aloe barbadensis*	Gwarpatha
33. *Mucuna prutita*	Konch
34. *Terminalia bellerica*	Baheda
35. *Tamarindus indica*	Imli
36. *Azadirachta indica*	Neem
37. *Achyranthes aspera*	Aandhijhara
38. *Barleria cacrulea*	Bajrandantis
39. *Barleria cristata*.	Badradantip
40. *Barleria prinoitis*	Bajradantip.
41. *Ocimum americanum*	Bapchii
42. *Centella asiatica*	Brahmibuti
43. *Datura metel.*	Dhatura
44. *Convolvulus arvensis*	Haranpadi
45. *Evolvulus alsinoides*	Shankhpushpi
46. *Cassia occidentalis*	Kasaundi
47. *Urginea indica*	Kolikanda
48. *Andrographis paniculata*	Kalmegh
49. *Helicteres ispara*	Marorphali
50. *Tinospora cordifolia*	Nimgiloy

Calotropis procera (Ait.) R.Br. (Akanda, Alarka, Aak) : The plant is one of the important numbers of traditional herbal medicine in every home of India. Traditionally the leaves of aak are warmed and tied around any body organ in pain. It is practically useful in backache and in joint pains. Warm leaves also relieve from stomach ache if tied around. Inhalation of burnt leaf cures headache. The traditional folk healers use the milky latex of aak for several ailments. Leaf latex if applied on fresh cut, stops bleeding immediately. Recent investigations have found that the alkaloids calotropin, calotaxein and uskerin are stimulant to the heart. Flowers and roots are used in Ayurvedic medicine. The plant is anthelmintic, the ashes act as an expectorant. The leaves are applied hot to the abdomen to cure the pain inside. The flower is tonic, antisialagogue, used as appetizer and against stomach ache, and cures piles and asthma. Flowers are believed to have detergent properties so they are given in cholera. The fresh roots are used as a toothbrush and are considered by pathans to cure toothache.. Alarka is an

alternative tonic and diaphoretic, in large dose emetic. Root bark is useful for treating chronic cases of dyspepsia, flatulence, constipation, loss of appetite, indigestion and mucus in stools. Leaves are used against guinea worms. Flowers are useful in asthma. Seed oil is geriatric and tonic. Green copra is given in asthma. Plant is used in spleen complaints, rheumatism, epilepsy, hemiplegia, sores, and smallpox and protracted labor.

Calotropis gigantea R.Br. (Arka) : Arka is purgative, anthelmintic alexipharmic,; cures leprosy, ulcers, leucoderma, tumors, piles, diseases of spleen, liver and abdomen. Juice is anthelmintic and laxative; cures piles and kapha. Dried and powdered plant is taken with milk and acts as a good tonic. Action is similar to Digitalis on the heart. Root bark and juice have emetic, diaphoretic, alternative and purgative properties. It is used in dysentery and as a substitute for Ipecauantha. It is regarded as a great remedy in syphilitic afflictions and is called "Vegetable mercury". In intermittent fevers it is used as antiperiodic and diaphoretic. It cures asthma and syphlis. In form of paste it is applied to elephantiasis. Tincture of leaves is used in intermittent fevers. Latex is bitter, heating, oleagenous and irritant, used in combination with *Euphrobia neerifolia* as purgative. Flowers are sweet, bitter, digestive, tonic, stomachic, anthelmintic, analgesic, astringent; cure inflamations, tumours, kapha and are good in ascites.

Jatropha curcas Linn. (Vyagrairanda) : Juice of Vyagrairanda is a well known purgative and is useful in whitlow, convulsions, syphilis, neuralgia, dropsy, anasarca, pleurisy and pneumonia. Root bark is applied externally in rheumatism and is used in sores. Leaves are galactagogue, rubefacient, suppurative, insecticidal and are used in foul ulcers, tumors and scabies, given internally in jaundice. Leaves are locally applied to breasts to increase secretion of milk. Leaves warmed and rubbed with castor oil and applied to boils and abscesses have supportive effect. Decoction of leaves is against diarrhoea, useful in stomach-ache and cough and also used for gargle to strengthen gums. Fresh stems are used as toothbrush. Fresh viscid juice flowing from stem is employed to arrest bleeding or hemorrhage from wounds. Stem bark is used for wounds of animal bites. Fruits and seeds are anthelmintic, useful in chronic dysentery, urinary discharges, abdominal complaints, anaemia, biliousness, fistula, and diseases of heart. Seeds are acro-nacrotic, poisonous to human beings and cattle and used against warts and cancers and also to promote hair growth. Seeds and oil are purgative, more drastic than castor oil. Wood causes dermatitis. Drug is bitter, acrid, astringent and anthelmintic. It serves to cleanse the entire system through its purgative property. It is useful in chronic dysentery, thirst, abdominal complaints, biliousness, anemia, fistula, ulcer, and diseases of the heart and skin.

Croton tiglium Linn. (Jamaalagotta, Jayapala) : Jayapala seeds and oil are drastic purgative, diaphoretic, vasicant, vermifuge irritant, rubefaceint and cathartic. Its action is prompt. Croton oil when rubbed on skin acts as a rubefaceint and counter-irritant and vesicant. When administered internally it operates as a powerful hydrogue cathartic. It is found to be very useful in ascites, anascara, cold, cough, fever, asthma, constipation, calculus, dropsy and enlargement of abdominal viscera. It is given only when a drastic purgative is required as in dropsy and cerebral affections like convulsions, insanity and

other fevers, attended with high blood pressure. Wood is diaphoretic in small doses and purgative and emetic in large doses.

Euphorbia hirta Linn. (Dudhi, Cara) : Cara is demulcent, antispasmodic, anti-asthmatic pectoral, anthelmintic and local parasiticide. Plant is chiefly used in the affections of childhood, in worms, bowel complaints and cough, in postnatal complaints, failure of lactation, breast pain. Extract of plant has depressant action and action on cardiovascular system, a sedative effect on mucous membranes of the respiratory and urino-genitory tract. Juice of plant is given in dysentery and colic, and milk applied to destroy warts. Plant alkaloid is effective in respiratory system and produces dilation of bronchi. Decoction of plant is used in bronchial affections and asthma. Latex is vermifuge and used in diseases of urino-genitory tract and also in application for warts.

Euphorbia tirucalli Linn. (Vajraduhu, Satsala) :It is useful in biliousness, leucorrhoea, leprosy, dropsy, whooping asthma, enlargement of spleen, dyspepsia, jaundice, colic tumours, and stones in bladder. Milky juice is vesicant and rubifacient. In small doses a purgative but in large doses it is acrid, emetic and counter-irritant; application for warts, neuralgia, rheumatism, toothache, asthma, cough and earache. It is also a fish poison. Milky juice is applied to itch and scorpion bites. Decoction of tender branches and that of roots is administered in colic and gastralgia.

Anti-HIV agents among desert plants

Around 40million people are affected due to the Human Immuno-deficiency Virus globally. During the past decades, a large number of anti-viral screening experiments on medicinal plant extracts have been reported and have led to the selection of several extracts active towards herpes viruses. A promising result of a naturally occurring antiherpetic agent was given by n-docosanol (a natural 22 carbon saturated fatty alcohol) which is undergoing phase III clinical trials in patients. Clinical testing of the topical formulation, or systemic administration of drug suspensions has demonstrated a good therapeutic index, since high doses of n-docosanol do not elicit appreciable toxicity. The findings show that natural products are still potential sources in the search for new antiherpatic agents(Hattori et al., 1995,). Various plant extracts used in Ayurvedic medicine for inhibitory effects on HIV virus have been studied (Hattori et al., 1993 ; Kusumoto et al., 1995; Hattori, personal communication). A large number of such plants occur in semi-arid and arid climate of Rajasthan (Roy and Kumar, 1995) .

Acquired immunodeficiency syndrome (AIDS) , the great pandemic of the second half of the 20th Century, is still a threatening disease world wide. Many research approaches are currently aimed at developing novel agents to arrest the replication of HIV through various targets. These may include the inhibition of reverse transcriptase (RT), protease (PR), membrane fusion and integrase. HIV PR enzyme has been demonstrated to play an essential role in viral replication (Meek et al., 1990). It is considered as potential target for anti-AIDS therapy, as the inhibition of this enzyme produces immature, non-infectious virions (Mous et al., 1988; Huff, 1991; Robins and Platter, 1993). A range of

HIV PR inhibitors have been designed and applied in clinical trials such as Sanqunavir, Ritonavir and Indinavir. However, the development of drug resistance by virus, irrespective of the target, remains as an overwhelming problem in AIDS chemotherapy (El Farrash et al. 1994). Thus there is great need to search for and develop new and different anti-HIV candidates from plants and natural products are of considerable importance.

In search for anti-HIV active agents from natural products, many attempts at screening traditional medicines have been made (Chang and Yeung, 1989; Otake et al., 1995; Wan et al., 1996). However Indian and other tropical region plants with their vast diversity, have not been investigated for their antiviral activity. Hussein et al. (1999) investigated forty eight methanol extracts from Sudanese plants which were screened for their inhibitory activity on viral replication. Nineteen extracts showed inhibitory effects on HIV–induced cytopathic effects (CPE) on MT-4 cells. The extracts were further screened against HIV-1 protease (PR) using an HPLC assay method. Of the tested extracts, the methanol extracts of the desert plants *Acacia nilotica* (bark and pods), *Euphorbia granulata* (leaves) , *Maytenus senegalensis* (stem-bark) and aqueous extracts of *A. nilotica* (pods) and *M. senegalensis* (stem-bark) showed considerable inhibitory effects against HIV-1 PR (Hussain et al., 1999) . Some of the plants from Sudan are common within the Indian dessert region of Rajasthan and generally they grow on the wastelands. They have potential use as bio-energy plantations (Kumar et al., 1995; Kumar, 1998). However a large number of them are used in the medicines of Ayurveda. They were also found effective against HIV-1. (Hussein et. al., 1999) . A list of potential plants of this region is given here in table 1. However these plants have not been studied in detail and there is need to study them for their medicinal properties including anti-HIV properties. Some of the active principles against anti-HIV are triterpenoids which are abundant in laticiferous plants of Rajasthan. Besides, *Ganoderma* sp is very frequently met in Rajasthan attacking trees. *Ganoderma lucidum* has been described to contain triterpenes which have inhibitory effects against HIV-1 protease (Min et al., 1998). Besides this, several other plants like *Abrus precatorius* L., Leguminosae (Chao-mei et al., 1998) , *Datura stramonium* L., *Balanites aegyptiaca* L. Delile etc. commonly found in Rajasthan show anti-HIV activity (Kawahata et al., 1996). In China, its seeds have been used as an insecticide and for skin diseases since ancient times .

A detailed survey of medicinally important plants has been carried out and important trees, shrubs and herbs have been listed and their characters studied in several publications from our laboratory. They included drugs for cure of urinary tract infection (Ajanta and Kumar, 2000b) anti-depressant herbal drugs (Ajanta and Kumar, 2000c), medicines for skin diseases (Shivani and Kumar, 2000), anticancer drugs (Sharma and Kumar, 2000); anti-diabetic drugs (Ritu and Kumar, 2000). Herbal drugs of Leguminosae from Rajasthan have been studied (Sapna and Kumar, 2000). Herbal crude drugs for anti-malaria (Anita and Kumar, 2001); anti-paralytic (Vandana and Kumar, 2001). Besides this, herbal crude drugs for cure of hepatic diseases (Santosh and Kumar, 2001) and diseases of the digestive system (Mamta and Kumar, 2001) have been studied for their characters and investigations on their morphological and pharmacognostical characterization are in progress.

Conclusion

The sustainable land utilization in the ecologically fragile climate of semi arid and arid regions has to be guided by the principal of optimal utilization of resources. It is a matter of great interest that a large number of plants of the arid and semi-arid regions of the world are effective as anti-HIV agents. They are also used in variety to herbal and traditional medicines as listed in this paper. Our previous work on their bio-energy production potential, if combined with their crude drug potential could yield bio-fuels on one hand and valuable crude drugs on the other. However a large number of tropical plants have not been studied in detail for their chemical constituents, pharmacological properties of the extracts, and their pharmacognostical characterization including DNA sequencing etc. If a joint collaboration could be established in this direction, valuable information could be generated with wide ranging practical applications. This could also provide alternative land use pattern for the rural poor thriving on marginal lands on one hand and help in eco-restoration on the other. The use of bio-energy plants in the herbal crude drugs has great potential and detailed investigations are planned with the help and cooperation of different agencies. This paper provided a brief outline of the work in the area for future suggestions and improvement.

Acknowldegement

I am deeply indebted to Professor Watanabe, Director of the Research Institute of Wakan Yaku for providing me an opportunity to work as visiting Professor at the Institute and Dr. Komatsu for constant encouragement support during my stay and work at the TMPU. Toyama.The keen interest and encouragement by Professor Dr. Neumann during the course of this work is gratefully acknowledged.

References

Arora, R.K. and E.R. Nayar, (1984): Wild relatives of crop plants of India. NBPGR. Science Monograph No. 9, New Delhi.

Byung-Sun, M., N. Nakamura and H. Miyashiro (1998): Triterpenes from the spores of *Ganoderma lucidum* and their inhibitory activity against HIV-protease. Chem. Pharm.Bull. 46 (10), 1607-1612.

Chang, R.S. and H.W. Young (1989): Inhibition of the growth of human immunodeficiency virus in vitro by crude extracts of Chinese medicinal herbs. Antiviral Res. 9, 163-176.

Chao-mei, M., N. Nakamura and M. Hattori (1998): Saponins and C-Glycosyl Flavones from the seeds of Abrus precatorius. Chem. Pharm. Bull. 46 (6), 982-987.

Choudhary, M. and A. Kumar (2001): Ayurvedic crude drugs for cure of diseases of the digestive system. Int. J. Mendel. 18, 27-28.

El-Farrash, M.A., M.J. Kuroda and T. Kitazaki, et. al. (1994): Generation and characterization of a human immunodeficiency virus type 1 (HIV-1) mutant to an HIV-protease inhibitor. J. Virol. 68:233-239.

Gupta R. and Kumar, A. (2000): Ayurvedic crude drugs as potential cure of diabetes. Int. J. Mendel. 17(3-4), 127-128.

Hattori, M., I.T. Kusumoto, M. Soga and T. Namba (1993): Screening of various Ayurvedic medicines for their inhibitory activities on the reverse transcriptase and Identification of arecatanins, an embelin, as major inhibitory substances from Areca catechu and Embelia ribes. J. Med. Pharm. Soc. Wakan-Yaku 10, 141-148.

Hattori, M., T. Nabakayashi and Y. Lim et al. (1995): Inhibitory effects of various Ayurvedic and Panamian medicinal plants on the infection of Herpes simplex virus 1 in vitro and in vivo. Phythother. Res. 9, 270-276.

Huff, J.R. (1991): HIV-1 protease: A novel chemotherapeutic target for AIDS. J. Med. Chem. 34, 2305-2314.

Hussein, G., H. Miyashiva, N. Nakamura, M. Hattori et al. (1999): Inhibitory effects of Sudanese plant extract on HIV-1 replication and HIV-1 Protease. Phytotherapy Res. 13, 31-36.

J.R.Goodin and D.K. Nottington (eds.). International Center for Arid and Semi-arid land studies, Lubbock, Texas, pp. 261-281.

Kawahata, ,T., T. Otake, H. Mori, M. Morimoto, N. Ueba, I.T. Kusumoto, S. El-Mekkawy, M.Hattori, T. Namba (1995): J. Trad. Med. 13, 59-65.

Kotia, A. and A. Kumar (2001b). Some of the common weeds of medicinal value from Rajasthan . Int. J. Mendel. 18, 17-20.

Kotia, A. and A. Kumar (2001a): Characterization of weeds on wastelands and their role in eco-development . Int. J. Mendel. 18, 7-8.

Kumar, A. (1998): Biomass energy crops of semi-arid regions of India and their energy potential. In: Biomass for Energy and Industry (H. Kopetz et al., eds.) CARMEN, Germany. pp 345 – 348.

Kumar, A. , S. Johari, and S. Roy (1995): Production and improvement of bioenergy sources. J. Indian Bot. Soc. 74A, 233-244.

Kumar, A. (2000): Hydrocarbon yielding plants and future prospects: Biotechnological approach. In: Plant Biotechnology (P.C. Trivedi, ed.) Panima. New Delhi. pp 194-212.

Kusumoto, T. Nakabayashi H. Kida et al. (1995): Screening of various plant extracts used in Ayurvedic medicine for inhibitory effects on human immunodeficiency virus type 1 (HIV-1) protease. Phytother. Res. 9, 180-184.

Meek, T.D., D.M. Lambert, B.W. Metcalf, S.R. Petterway and G.B. Dreyer (1990): HIV-1 protease as target for potential anti-AIDS drugs. In: Design of Anti-AIDS Drugs. E. DeClerckq,ed. Elsevier Science Publishers B.V. , The Netherlands, pp. 225-256.

Mishra, A. and A. Kumar (2001b): Studies on Ayurvedic crude drugs for the cure of urinary tract stones. Int. J. Mendel 18 (1-2), 41-42.

Mishra, A. and A. Kumar (2001a): Medicinally important trees of Rajasthan. Int. J. Mendel. 18, 37-38.

Mishra, A. and Ashwani Kumar (2001c): Searching for anti-depressant crude drugs in ayurvedic system of medicine. Int. J. Mendel. 17 (3-4), 91-92.

Mous, J., E.P. Heimer and S.F.J. Le Grice (1988): Processing protease and reverse transcriptase from human immunodeficiency virus type 1 polyprotein in Escherischia coli. J. Virol. 62, 1433-1444.

Otake, T., H. Mori, M. Morimoto et al. (1995): Screening of Indonesian plant extracts for anti-human immunodeficiency virus type 1 (HIV-1) activity. Phytother. Res. 9, 6-10 .

Paroda, R.S. (1979): Plant resources of Indian arid zones for industrial uses. In: Arid land Plant resources.

Robbins, T. and J. Platter, (1993): HIV protease inhibitors, their anti-HIV activity and potential role in treatment. J. AIDS. 6, 162-170..

Roy, S. and A. Kumar (1995): Biodiversity of Rajasthan and its energy potentials. J. Enviroment and pollution. 2(3), 105-109.

Sanghi, S. and A. Kumar (2000): Characterization of some of the medicinal plants of family Leguminosae used in Ayurvedic system of medicines. Int. J. Mendel. 17, 109-110.

Sharma, L.K. and A. Kumar (2000): Searching for anti-cancer drugs in traditional medicines. Int. J. Mendel. 17, 77-78.

Sharma, S. and A. Kumar (2001): Ayurvedic plants for cure of hepatic diseases. Int. J. Mendel. 18: 13-14.

Sharma, V. and A. Kumar (2001): Studies on anti-paralytic crude drugs used in Ayurvedic system. Int. J. Mendel. 18, 33-34.

Shivani and A. Kumar (2000): Ayurvedic medicinal plants used for skin diseases. Int. J. Mendel. 17, 105-106.

Wan , M., S. Bloor, L. Foo and B. Loh (1996): Screening of New Zealand plant extracts for inhibitory activity against HIV-1 protease. Phytother. Res. 10, 589-595.

Yadav, A. and A. Kumar (2001): Studies on anti-malarial drugs used in Ayurvedic system. Int J. Mendel Int. J. Mendel. 18(1-2), 29-30.

Rubisco; easy Purification and Immunochemical Determination

Ulrich Groß

Justus-Liebig-Universität Gießen, Institute of Plant Nutrition, Department of Tissue Culture, Südanlage 6, D-35390 Giessen

e-mail: Ulrich.Gross@aranea.de

Abstract

Rubisco (Ribulose-1.5-bisphosphate carboxylase/oxygenase) from spinach was purified to homogeneity in one step by gel filtration. This enzyme is suitable for the generation of a specific antibody in rabbits. The enzyme concentration in spinach leaves amounted to 40 % of the total soluble protein. The specific antibody shows cross reaction with crude extracts from leaves of other higher plants. The enzymes subunits could be separated by denaturing preparative SDS gel electrophoresis.

Introduction

Rubisco (Ribulose-1.5-bisphosphate carboxylase/oxygenase) fixes globally about 10^{11} tons of carbon dioxide per year. The native enzyme consist of 8 large and 8 small subunits. In higher plants the large subunit is encoded by the chloroplast, whereas the small subunit is encoded by the nuclear genome. The small subunit is synthesised as a precursor protein in the cytoplasm and is transported into the chloroplast (Cornwell and Keegstra, !987). In red and brown algae both genes are encoded by the plastid DNA and are transcribed bicistronically (Bohnert and Jensen, 1988). The primary structure of the large subunit is conservative, whereas the amino acid composition of the small subunit varies in the plant kingdom. Thus, the large subunit has a molecular weight of about 55 kilo Dalton, whereas the small subunits amounts to 12 to 14 kilo Dalton in green plants (Parry et al., 1987).

Material and Methods

The activity of Rubisco was determined by incorporation of $H^{14}CO_3^-$ into acid stable products according to the method of Groß et al. (1993). Total soluble protein was measured by the method of Esen (1978). Partially purified Rubisco from spinach was purchased from Sigma and purified by gel chromatography with Sephacryl S400 (97·2.6 cm, Pharmacia) in 50 mM Tris-HCl, pH 8 and 50 mM NaCl at 4 °C. The flow rate was 24 ml · h^{-1} and fractions of 9.5 ml were collected. The recorder speed was 5 mm· h^{-1}· The gel was checked beforehand by chromatography of 50 mg cytochrome c. SDS-gel electrophoresis was carried out according to Laemmli (1970) using an acrylamid content of the separating gel of T = 12.5%. Specific antibodies against purified Rubisco were generated as described by Groß et al. (1993). Rubisco concentration was measured by positive ELISA according to the method of Catt and Millard (1988). The calibration curve for this assay was ranged from 0.01 to 1 µg · ml^{-1}.

Double radial immundiffusion was carried out as reported by Ouchterlony (1958). The subunits of rubisco were separated by preparative denaturing gel electrophoresis and eluted electrically from the gel (Groß, 1990).

Results

Partially purified powder of Rubisco from spinach can be bought from the chemical trade. It is available in large amounts as a lyophilised powder with stable activity. This enzyme can easily be purified about 7-fold by gel filtration (table 1). The exclusion limit of the column was clearly separated from the separation limit. Rubisco eluted from fraction 35 to 40 within the separation limit with the main protein peak (figure 1). The enzyme was more or less electrophoretically pure (figure 2) and was used for the production of a specific antibody in rabbits. The antibody produced a single band against leave extracts from spinach. It showed cross reactivity with leaves from other higher plants like peanut, carrot and the purified enzyme from peanut leaves (figure 3). No reactions occurred in diffusion tests against preimmuno serum (data not shown). Rubisco concentration from spinach leaves was determined by ELISA. It amounted to 400,4 ± 4,4 µg per mg total soluble protein (mean ± SD, n = 3).
Rubisco subunits were purified and separated from each other under preparative denaturing conditions. Figure 4 shows the results of this procedure. The two proteins were applied to different lanes of an analytical electrophoretic gel. The large subunit was applied to lane 2, and the small subunit to lane 3. The two parts of the native protein (lane 1) occurred in different lanes without any contamination of the other portion.

Table 1: Purification of Rubisco from spinach leaves by gel filtration on Sephacryl S400

	volume [ml]	protein [mg·ml^{-1}]	specific activity [nkat·mg^{-1}]	purification factor
Rubisco (Sigma)	1.3	75	0.76	1
Rubisco (purified)	57	0.82	5.63	7.4

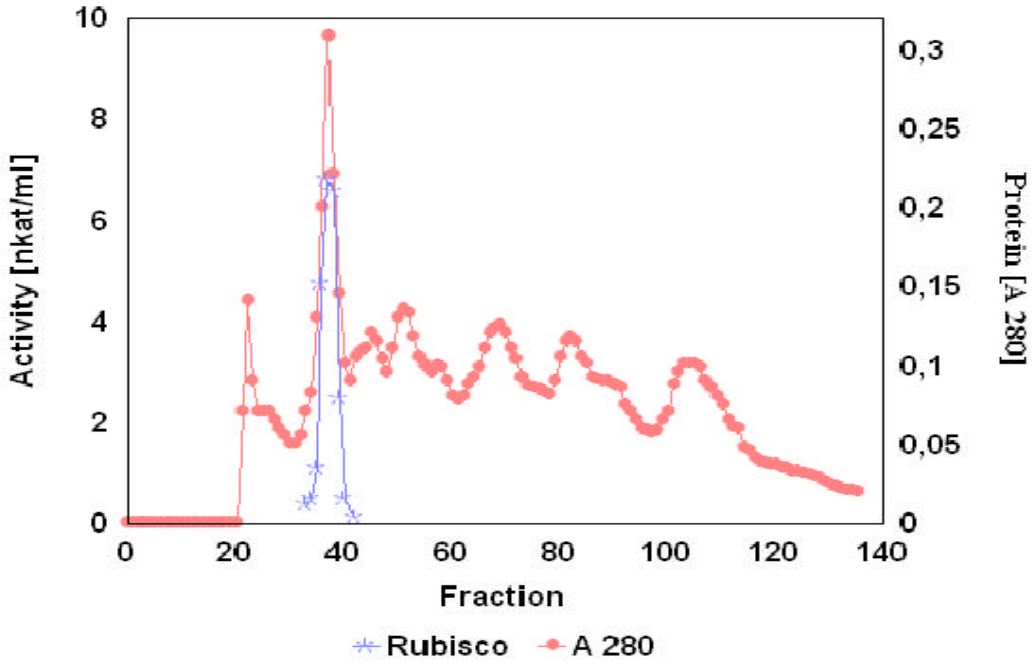


Figure 1: Elution profile of rubisco from spinach leaves on Sephacryl S400; A 280 = absorbance at 280 nm.

Figure 2: SDS-gel electrophoresis of purified rubisco from spinach (30 µg total soluble protein); LSU = large subunit, SSU = small subunit.

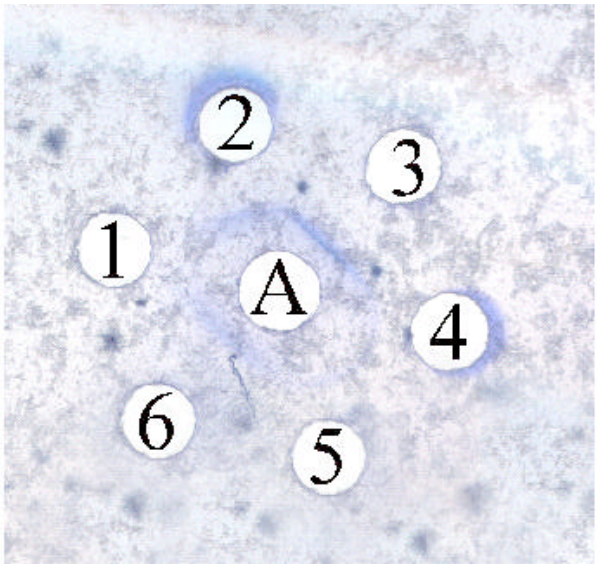


Figure 3: Double radial immunodiffusion: 1, H_2O; 2, crude extract from spinach leaves (4,48 mg ml $^{-1}$ total soluble protein); 3 and 6, crude extract from peanut leaves (6,41 mg ml $^{-1}$ total soluble protein); 4, purified rubisco from peanut leaves (0,7 mg ml $^{-1}$ total soluble protein); 5, crude extract from carrot leaves (3,51 mg ml $^{-1}$ total soluble protein); A, specific antibody against Rubisco from spinach.

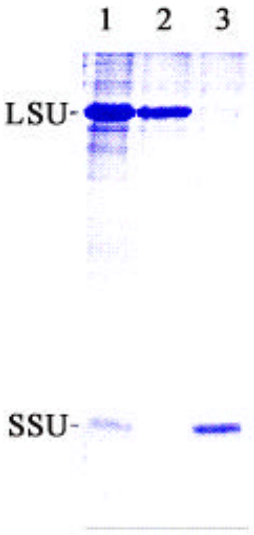


Figure 4: SDS gel electrophoretic pattern of partially purified rubisco from spinach (Sigma) and its separated subunits: 1, partially purified rubisco from spinach purchased from Sigma (30 µg total soluble protein); 2, large subunit (3 µg total soluble protein); 3, small subunit (10,5 µg total soluble protein); LSU = lage subunit, SSU = small subunit.

Discussion

Rubisco activity is regulated by pH and Mg^{2+} concentration. This means that a constant enzyme concentration can vary in ist activity. Furthermore, it has been reported in the literature that a sucrose supplement to the nutrition medium of photoautotrophic peanut cultures reduces rubisco activity without affecting its concentration (Groß et al., 1993). Thus, it is important to measure both enzyme activity and its concentration.
Rubisco from spinach was purified by an easy procedure. This preparation is suitable for the generation of a specific antibody in rabbits. A single precipitation line in an Ouchterlony immunodiffusion assay against crude extract from spinach leaves confirms the specificity of the antibody. Using this antibody, semi- or fully quantitative measurements of rubisco concentrations can be performed according to the method of Catt and Millard (1988). Furthermore, Rubisco obtained from other plant species can easily be purified by affinity chromatography using the immobilized antibody against spinach rubisco.
The two subunits of rubisco were separated and purified by preparative gel electrophoresis. This technique represents a means to investigate the interaction of the chloroplast and nuclear genome by concentration measurement of the enzyme subunits employing the specific antibody against the whole enzyme. Moreover, specific antibodies against the isolated subunits can be easily obtained by immunizing rabbits with the single proteins.

References

Bohnert, H. J. and R. G. Jensen (1988): Rubisco ’87: Anything new? Plant Molecular Biology Reporter 6, 32-39.

Catt, J. W. and P. Millard (1988): The measurement of ribulose-1.5-bisphosphate carboxylase/oxygenase concentration in the leaves of potato plants by enzyme linked immunosorbent assays. J. Exp. Bot. 39, 157-164.

Cornwell, K. L. and K. Keegstra (1987): Evidence that a chloroplast surface protein is associated with a specific binding site for the precursor to the small subunit of ribulos-1,5-bisphosphate carboxylase. Plant Physiol. 85, 780-785

Esen, A.(1978): A simple method for quantitative, semiquantitative and qualitative assay of protein. Anal. Biochem. 89, 264-273.

Groß, U. (1990): Der Einfluß von Saccharose und der CO_2-Konzentration auf die Aktivität von Ribulose-1,5-bisphosphat Carboxylase/Oxygenase und Phosphoenolpyruvat Carboxylase und die Konzentration der Ribulose-1,5-bisphosphat Carboxylase/Oxygenase in photoautotrophen Zellkulturen von Arachis hypogaea L. und Daucus carota L. Ph.D. thesis, Justus Liebig - University Gießen (1990), Germany.

Groß, U., F. Gilles, L. Bender, P. Berghöfer and K.-H. Neumann (1993): The influence of sucrose and an elevated CO_2 concentration on photosynthesis of photoautotrophic peanut (Arachis hypogaea L.) cell cultures. Plant Cell Tiss. Org. Cult. 33, 143-150.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Parry, M. A. J., C. N. G. Schmidt , M. J. Cornelius, B. N. Millard, S. Burton, S. Gutteridge, T. A. Dyer and A. J. Keys (1987): Variations and properties of ribulose-1,5-bisphosphate carboxylase from various species related to differences in amino acid sequences. J. Exp. Bot. 38, 1260-1271

Ouchterlony, O. (1958): Diffusion-in-gel methods for immunological analysis. Prog. in Allergy 5, 1-78.

Genomic Instabilities in Tissue Culture – a Physiological Normality?

Birgit Arnholdt-Schmitt

Departamento de Bioquímica e Biologia Molecular, Universida de Federal do Ceará (UFC), 60001-970 Fortaleza, CE, Brasil

e-mail: arnoldt-schmitt@gmx.de

Abstract

In the present paper, the general role of in vitro–culture systems for fundamental research in plant sciences is stressed. As an example, studies on the effect of physiological stimuli on genome variation are presented. The results of systematic investigations on 37 primary callus culture variants derived from secondary phloem of carrot roots indicated a drastic diminution in repetitive DNA fractions as a predominant change at the total genome level during high cell division growth. Also, RAPD fingerprint analyses confirmed reproducible quantitative polymorphism in primary cultures due to the effects of tissue culture and growth regulators. The general implication of these results could be confirmed by in vivo - studies on different tissues and tissues of different ages.

Introduction

From the historical point of view, in vitro-culture techniques for plants were developed primarily to facilitate basic physiological research (Haberlandt, 1902). However, the search for commercial applications of these techniques started very early in the sixties and tied up personal and funding capacities in this area, so that fundamental research on in vitro cultures, as a complementing system to research on intact plants, became a stepchild in physiological investigations (Neumann, 1999). Nevertheless, in vitro-cultures of plant tissues proved to be especially worthwhile in revealing basic aspects of metabolic pathways (e.g. Steward et al., 1952; Gross et al., 1993), of plant growth and development (e.g. Grieb et al., 1994) and of differential genome variability (e.g. Nagl 1976, 1979, 1992; Schaefer et al., 1978; Duehrssen and Neumann, 1980; Cullis, 1987; Bassi, 1990; Arnholdt-Schmitt,

1995; Arnholdt-Schmitt et al., 1995; Bogani et al., 1996; Schaefer et al., 2000). In the present paper, recent results on physiological and differential genome variation obtained with the help of well–defined primary tissue cultures and analytical procedures using restriction enzymes and RAPD fingerprinting strategies will be given. Additionally, in vivo investigations on different tissues and tissues of different ages will be presented to demonstrate, as an example, the general relevance of results achieved by the integration of in vitro – cultures as model systems in basic research.

Material and Methods

Plant material and primary callus culture

Carrot plants from the local market and of the cultivars Rote Riesen, Rotin, Lobbericher and Pariser Markt were used. For in vivo analyses, parts of the xylem and secondary phloem tissue of individual plants were isolated from the tap root. Blades of young and older leaves were harvested separately. For in vitro experiments, primary callus cultures were initiated from the secondary phloem of carrot roots taken from plants of different ages. 5 explants of 2 to 4 mg isolated from the secondary phloem next to the cambial ring were cultured in liquid medium (Neumann, 1966) in T-tubes (Steward et al., 1952) which were continuously rotated at 1 rpm under permanent illumination (approximately 4000 lux, osmium lumilux white) at 21°C and 28°C.

DNA isolation and restriction analysis

High-molecular weight DNA was extracted employing the method described by Murray and Thompson (1980) which was later simplified by Power et al. (1986). Removal of RNA was obtained by treatment with ribonuclease A and T1. Further purification of the extracts was achieved by using chloroform-isoamylalcohol. Quantification of the DNA was performed chemically employing the diphenylamine reaction (Richard, 1974). Genomic DNA was digested completely by the methylation-insensitive restriction enzyme *Bst*NI (Biozym diagnostic) using 3 to 4 U/µg DNA for at least 4 hours. The recognition sequence is 5'-CC(AT)GG. Electrophoresis was performed with 2 to 3 µg of DNA fragments in 1 % agarose (Bio-Rad Standard Low-*Mr*) for 15 hours (50 V at 15 °C) and results were documented photographically (Kodak Tri-X Pan, 400 ASA) using a transilluminator and light of 302 nm. Scanning of the restriction fragments was carried out on the negatives. To level out differences between the amount of DNA in the different variants with the amount actually loaded onto the gel, the integral of the sum of all fragments distributed as bands and background in the gel of one variant was corrected to the total integral of the DNA fragments of the compared variant.

DNA isolation and RAPD fingerprinting

DNA isolation for RAPD analyses was performed by employing the DNeasy Plant Mini Kit from Qiagen (Hilden, Germany). Extracted DNA was checked for quality and quantity with the help of a video densitometer in comparison to lambda DNA in 1 % agarose. 5 to 10 ng DNA was used for RAPD analysis in 25 reaction volumes. The PCR was performed with RAPD analysis kits (Ready-to-go RAPD beads, Amersham Pharmacia Biotech, Freiburg, Germany) in a Techne thermocycler (model Progene). 10mer random primers were obtained from Roth (Karlsruhe, Germany) or from Amersham Pharmacia Biotech (Freiburg, Germany). Conditions for the PCR reaction were as follows: 5 min at 95 °C, 45 cycles at 95 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min; for final extension 5 min at 72 °C. RAPD fragments were separated in 1.5 % agarose (Gibco-BRL). The method applied was carefully checked for good repeatability which included repeated extractions, PCRs and electrophoreses, the use of various thermocyclers and the effect of different personnel working with the method.

Results

Digestion of the DNA of the secondary phloem of carrot roots by the restriction enzyme *Bst*NI characterizes the *Daucus carota* genome by a distinctive pattern of fragments. As shown by the video densitometric scan of the DNA of cv. Rote Riesen in Fig. 1A, at least 19 bands of repetitive fragments can be distinguished. Analysis of thirty-one individual plant genomes, including plants of cv. Rote Riesen, cv. Rotin, cv. Lobbericher, cv. Pariser Markt and an unknown genotype from the local market displayed the same pattern of restriction fragments, indicating a general characteristic of the *Daucus carota* genome (data not shown, see Arnholdt-Schmitt, 1993). Initiation of primary cultures from explants of the secondary root phloem of various carrot genotypes at different ages in the presence of myo-inositol (0.28 mM), IAA (11.42 µM) and kinetin (0.47 µM) at 21°C and 28°C induced callus growth, that was mainly due to cell division activity ($r = 0.81$). The mass of actually produced callus varied significantly between 14.2 mg and 161.3 mg, which, in a complex manner, was due to different factors that were included in the study (see Arnholdt-Schmitt, 1999). Two weeks after inoculation, a drastic diminution in the intensity of the bands of repetitive fragments was observed, as is shown as an example for cv. Rote Riesen in Fig. 1B. This change in genome organisation was found to take place in all of the 37 investigated growth variants and, as though, seems to occur independently of the carrot genotype, the age of plants used for the experiments, the growth temperature (21°C or 28 °C), and the actual callus growth intensity. This phenomenon, which indicates a transient elimination of repetitive DNA fractions during callus growth induction (for detailed discussion see Arnholdt-Schmitt, 1995), was checked for confirmation by applying RAPD analysis on comparable primary cultures. Fig. 2 shows the RAPD pattern of the genomic DNA of explants isolated from the secondary root phloem of 3 individual plants of an unknown carrot genotype at t_0 and three weeks after initiation of a primary culture. A putative loss of a band of 1570 bp was observed in cultured explants taken from all three

plants. By varying the relationship between primer and template DNA, it could be demonstrated, that the band of 1570 bp was existent at a minor level also in the primary culture, but was below the detection limit, when standard conditions were applied (data not shown; for method see Schaffer and Arnholdt-Schmitt, 2001; Schaffer, 1999; Schäfer et al., 2000). Fig. 3 indicates, that growth regulators may be involved in the induction of polymorphism in genomic DNA. At the 14^{th} day of primary culture, a distinctive, amplified fragment of 976 bp was clearly visible, if secondary root phloem explants were cultured without any growth regulators or with myo-inositol and IAA only, but was not evident any longer, when kinetin was supplied in addition to myo-inositol and IAA. By varying the reaction conditions for primer and template DNA concentration, this putative loss of a fragment could again be deduced to a merely quantitative effect on the number of fragments produced by the chosen primer (data not shown, see Schäfer et al., 2000).

To analyse, whether this kind of quantitative polymorphism in the genome, which was induced by the defined in vitro–culture conditions and which was accompanied by a change of the physiological and differentiational state of the tissue, would be significant also under in vivo conditions, different tissues of carrot plants as well as leaves of different age were analysed by RAPD fingerprinting. Fig. 4 demonstrates the pattern of amplified fragments produced by primer OP-G06 of the DNA of xylem tissue (lane 1) and of the secondary phloem (lane 2) of carrot roots, the blade of young leaves (lane 3), the blade of older leaves (lane 4) and a mixture of petioles from younger and older leaves (lane 5). The RAPD profiles of the root tissues (xylem and secondary phloem) were identical regarding the occurrence as well as the intensity of bands. This could also be confirmed by additional primers (data not shown, see Schäfer, 1999). By comparing these patterns of the root material with that of the leaves, a fragment of 1050 bp (white arrow) is visibly lost in older leaves and remarkably diminished in its amount in young leaves as compared to the root tissues. In repetitive experiments it became clear that, in fact, the putative loss of the 1050 bp fragment in older leaves was based again on a quantitative polymorphism, which obviously kept the amplified fragment from older leaves around the detection limit. Additionally, 5 bands of the DNA of older leaves display a significantly reduced intensity (black arrows) also in comparison to younger leaves, whereas two fragments show a higher rate of amplification (white circles) in older leaves. Band intensities in the pattern of the petioles may serve as a control variant for the method, since this sample was a mixture of younger and older tissues which is also mirrored by intermediate band intensities in comparison to younger and older leaves. Without the necessity of screening a great number of primers, this tendency of decreased band intensities in the fingerprints of older leaves was easily confirmed by the use of only some additional primers (data not shown, see Schäfer, 1999).

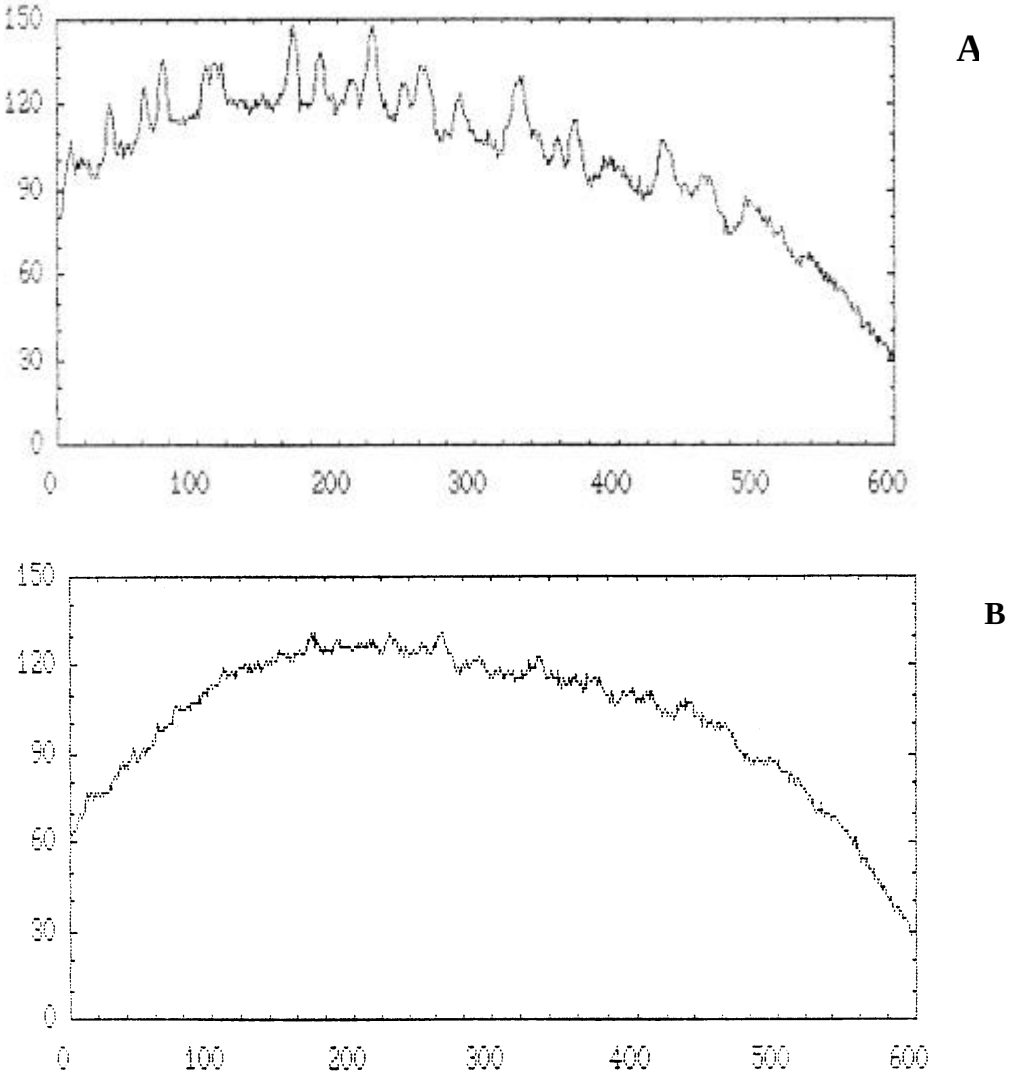


Figure 1: Restriction enzyme analysis with *Bst*NI of the genomic DNA of secondary root phloem explants of cv. Rote Riesen at t_0 (**A**) and 14 days after primary culture induction in the presence of myo-inositol, IAA and kinetin (**B**)

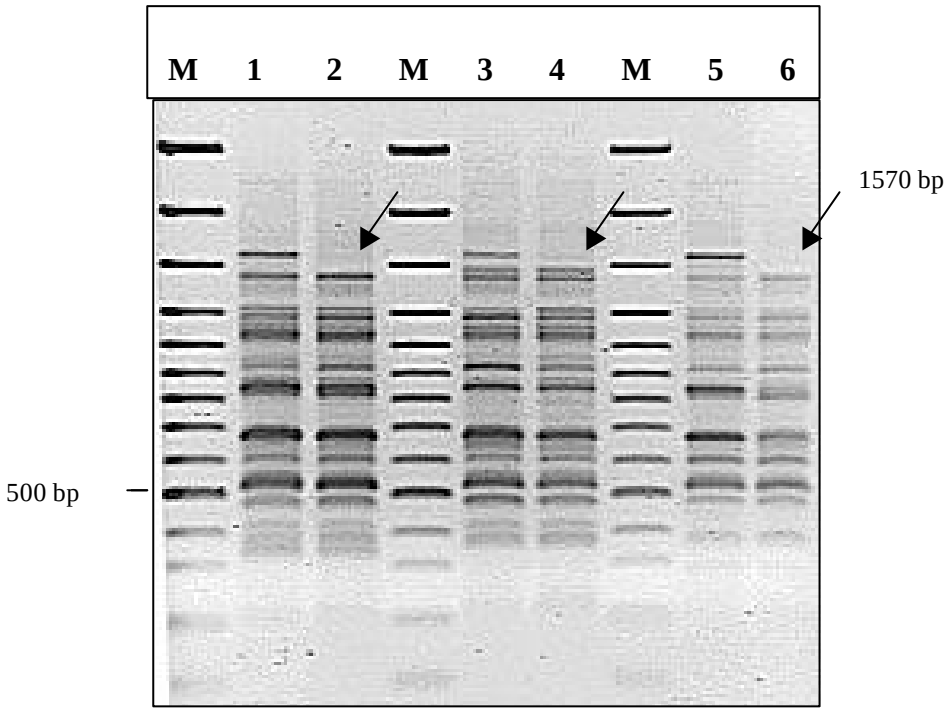


Figure 2: RAPD fingerprints of carrot secondary root phloem explants isolated from three individual plants at t_o (lane 1, 3, 5) and 21 days after inoculation (lane 2, 4, 6). M = marker. Primer: OP-D08 5´-GTGTGCCCCA-3´ (see Schaffer, 1999 and Schäfer et al., 2000)

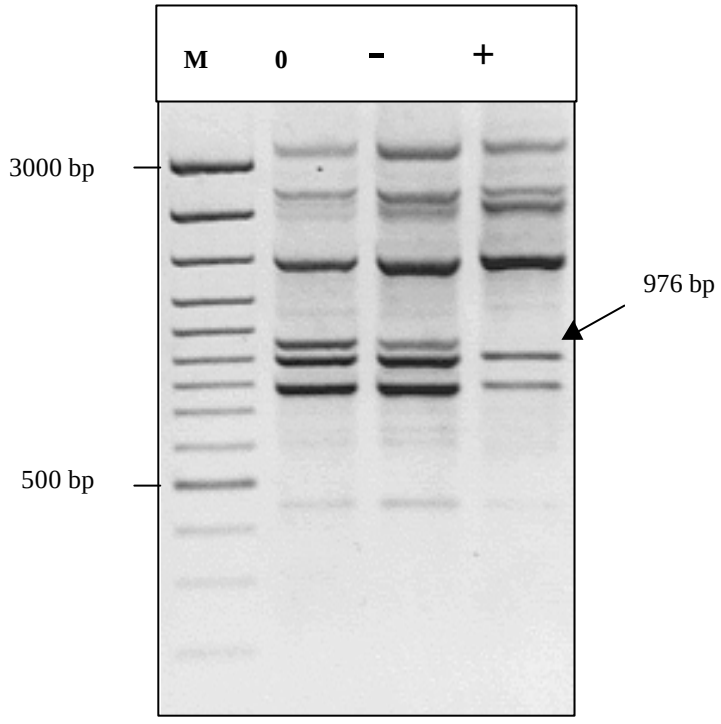


Figure 3: RAPD fingerprints of secondary phloem explants isolated from carrot roots 14 days after inoculation without hormones (0) or in the presence of myo-inositol and IAA without (-) or with (+) kinetin. Primer: P2 5´-GTTTCGCTCC-3´ (see Schaefer et al., 2000)

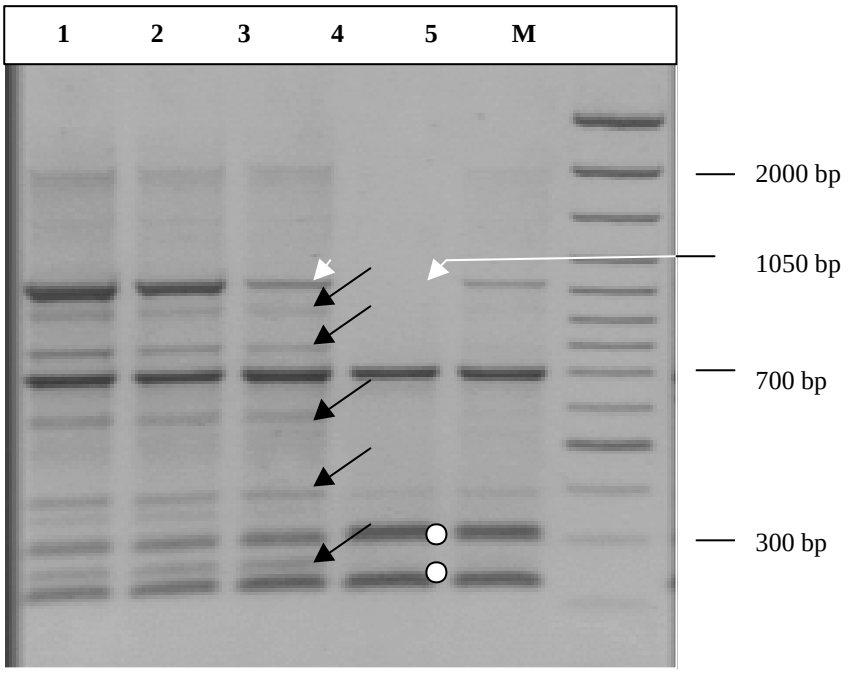


Figure 4: RAPD fingerprints produced by primer OP-G06 of various tissues of a carrot plant.(1) xylem of the tap root, (2) secondary phloem of the tap root, (3) young leaf, (4) older leaf, (5) petioles of young and old leaves.
M = marker (see Schäfer, 1999)

Discussion

A great benefit of in vitro-culture techniques to basic physiological and biochemical research on plants is given by the possibility to induce dynamic adaptation of plant cells or tissues to well-defined chemical and physical conditions. Insights gained by this strategy are appropriate to reveal potential capabilities of plant cells and / or tissues to respond to physiological stimuli. Although, of course, the complexity of the whole plant organism in its environment has to be considered, this strategy in research helps to check objectively for the significance of potential pathways in a given situation. In the present paper, systematic investigations on a great number of short term in vitro-cultures of carrot root explants are reported, which demonstrate, that a change in the physiological and differentiational state of tissues is accompanied by non–random quantitative polymorphism at the total genome level. This kind of plasticity occurred independently of individual genotypic differences between single plants of various carrot cultivars and seems to be more associated with typical characteristics of the carrot genome per se interacting with physiological stimuli. To test the general relevance of the results of in vitro–culture experiments for whole plant organisms, the genome of different tissues and leaves of different ages of carrot plants were checked employing RAPD fingerprint analysis for the occurrence of quantitative polymorphism. In-vivo investigations verified, that differentiation as well as ageing were accompanied by quantitative polymorphism, suggesting a determining role of gross changes in DNA organisation for tissue development (for detailed discussion of methods and results see Arnholdt-Schmitt, 1995; Schäfer et al., 2000; Schaffer and Arnholdt-Schmitt, 2001). Experiments are running to characterize sequences of RAPD fragments which are differentially amplified or lost as a response to physiological stimuli, inclusively nutritional factors like phosphorus (Imani et al., in prep (a), Imani et al., in prep (b)). Sequence informations gained so far indicate, that gene sequences as well as non–gene sequences could be affected by this kind of quantitative polymorphism (Imani et al., in prep (b); Schaffer, 1999). In conclusion, in vitro culture experiments were shown to be helpful in revealing systematically occurring quantitative changes in DNA organisation, that seem to be involved in plant adaptation as a response to physiological stimuli. The general ocurrence of quantitative polymorphisms in vivo in relationship to physiological events and differentiation could be confirmed with the applied fingerprint technique. Future experiments on ageing will reveal, whether the loss of band intensity shown by RAPD fingerprinting are related to the progressive loss of repeated sequences at the end of chromosomes (telomere shortening) observed during ageing (Shay and Wright, 1999). Since RAPD analysis enables screening for unknown sequences which are affected by quantitative polymorphism, future data will provide sequence information about differentially amplified or lost fragments related to physiological stimuli and, as though, are expected to contribute to the understanding of physiological and ecophysiological events.

Acknowledgements

The author is especially grateful to Prof. K.-H. Neumann for engaged discussions during a long cooperation, and for always providing laboratory space to perform research projects and to supervise students. Special thanks are also due to Claudius Schäfer and Sebastian Schaffer and their contribution to this paper by results obtained during the experimental work of their undergraduate thesis work and to Christa Lein for her competent and reliable technical assistance in tissue culture work.

References

Arnholdt-Schmitt, B. (1993): Hormonsensitivität und genomische DNS – Variabilität in ihrem Bezug zur Ertragsbildung bei *Daucus carota* L. ssp. sativus. Ein Beitrag zur Zellbiologie pflanzlichen Wachstums. Habilitation thesis, Justus-Liebig-University Giessen, Germany.

Arnholdt-Schmitt, B. (1995): Physiological aspects of genome variability in tissue culture. II. Growth phase-dependent quantitative variability of repetitive BstN I fragments of primary cultures of *Daucus carota* L. Theor. Appl. Genet. 91, 816-823.

Arnholdt-Schmitt B. (1999): On the physiology of yield production in carrots - Implications for breeding towards nutrient efficiency. Gartenbauwissenschaft 64 (1), 26-32.

Arnholdt-Schmitt, B, S. Herterich and K.-H. Neumann (1995): Physiological aspects of genome variability in tissue culture. I. Growth phase-dependent differential DNA methylation of the carrot genome (*Daucus carota* L.) during primary culture. Theor. Appl. Genet. 91, 809-815.

Bassi, P. (1990): Quantitative variations of nuclear DNA during plant development: A critical analysis. Biol. Rev. 65, 185-225.

Bogani, P., A. Simoni, P. Lio, A. Scalpi, M. Buiatti (1996): Genome flux in tomato cell clones cultured in vitro in different physiological equilibria. II. A RAPD analysis of variability. Genome 39, 846-853.

Chen, L.F.O., H.Y. Kuo, M.H. Chen, K.N. Lai, S.C.G. Chen (1997): Reproducibility of the differential amplification between leaf and root DNAs in soybean revealed by RAPD markers. Theor. Appl. Genet. 95, 1033-1043.

Cullis. C.A. (1987): The generation of somatic and heritable variation in response to stress. Am. Nat. 130, 62-73.

Dührssen, E. and K.-H. Neumann (1980): Characterization of Satellite-DNA of *Daucus carota* L. Z. Pflanzenphysiol. 100, 447-454.

Grieb, B., U. Groß, E. Pleschka, B. Arnholdt-Schmitt and K.-H. Neumann (1994): Embryogenesis of photoautotrophic cell cultures of *Daucus carota* L.. Plant Cell Tiss. Org. Cult. 38, 115-122.

Gross, U., F. Gilles, L. Bender, P. Berghöfer and K.-H. Neumann K. (1993): The influence of sucrose and an elevated CO_2 concentration on photosynthesis of photoautotrophic peanut (*Arachis hypogaea* L.) cell cultures. Plant Cell Tiss. Org. Cult. 33, 143-150.

Haberlandt, G. (1902): Kulturversuche mit isolierten Pflanzenzellen. Sitzungsber. Mat. Nat. Kl. Kais. Akad. Wiss. Wien 111, 69-92.

Imani, J., S. Schaffer, G. Langen and B. Arnholdt-Schmitt (a): Phosphorus depletion induces amplification of a sequence homologous to an intron of a leucin-rich repeat protein (LRR). (in prep). ACCESSION AJ311890 (http://www.ncbi.nlm.nih.gov/).

Imani, J. S. Schaffer, G. Langen and B. Arnholdt-Schmitt (b): Phosphorus-independent loss of a RAPD fragment homologous to the photosystem I P700 chlorophyll A apoprotein A1 during cell division growth of a primary culture. (in prep). ACCESSION AJ311807 (http://www.ncbi.nlm.nih.gov/).

Murray, M.G. and W.F. Thompson (1980): Rapid isolation of high–molecular-weight plant DNA. Nucleic Acids Res. 8, 4321-4325.

Nagl, W. (1976): Zellkern und Zellzyklen**.** Ulmer, Stuttgart.

Nagl, W. (1979): Differential DNA replication in plants: A critical review. Z. Pflanzenphysiol. 95, 283-314.

Nagl, W. (1992): Replication. Prog. Bot. 53, 166-180.

Neumann, K.-H. (1966): Wurzelbildung und Nucleinsaeuregehalt bei Phloem– Gewebekulturen der Karottenwurzel auf synthetischem Naehrmedium verschiedener Hormonkombinationen. Les Phytohormones et l'Organogenèse 38, 95 – 102.

Neumann, K.-H. (1999): Some physiological Aspects in the development of plant cell and tissue culture. Symposium in Memory of the 100th Anniversary of the Foundation of Tissue Culture by Gottlieb Haberlandt. 8-9 October 1998, Vienna. In Vitro Plant 35(2), 159-161.

Power, J.B., M.R. Davey, J.P. Freeman, B.J. Mulligan, E.C. Cocking (1986): Fusion and transformation of plant protoplasts. Methods Enzymol. 118, 578-595.

Richards, G.M. **(**1974): Modification of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. Anal. Biochem. 57, 369-376.

Schäfer, A., J.R. Blaschke and K.-H. Neumann (1978): On DNA metabolism of carrot tissue cultures. Planta 139, 97-101.

Schäfer, C. (1999): Untersuchungen zum Einfluß ontogenetischer Faktoren auf die DNA Organisation (RAPD-Analysen). Undergraduate thesis (Diplomarbeit), Justus-Liebig- University Giessen, Germany.

Schäfer, C., S. Schaffer and B. Arnholdt-Schmitt (2000): Differential RAPD fingerprints in carrot tissues. Acta Hort. 530, 437-445.

Schaffer, S. (1999): Zum Einfluß von Wachstumsstatus und der Nährstoffversorgung mit Saccharose und Phosphat auf den RAPD-Fingerprint eines Gewebes. – Untersuchungen an einer Primärkultur von *Daucus carota* L. Undergraduate thesis (Diplomarbeit), Justus-Liebig-University Giessen, Germany.

Schaffer, S. and B. Arnholdt-Schmitt (2001): Characterization of genome variation in tissue cultures by RAPD fingerprinting – a methodological comment. Plant Biosystems 135(1), 115-120.

Shay, J.W. and W.E. Wright (1999): Telomeres and telomerase in the regulation of human cellular aging. In: Molecular Biology of Ageing, Alfred Benzon Symposium 44. V.A. Bohr, B.F. Clark and T. Stevnsner (eds.) Munksgaard, Copenhagen. pp 148-158.

Steward, F.C., S.M. Caplin and F.K. Millar (1952): Investigations on growth and metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition and growth in undifferentiated cells. Ann. Bot. 16(1), 57-77.