In vitro germplasm preservation through regenerative excised root culture for conservation of phytodiversity

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In vitro preservation of germlasm becomes imperative for those plant species in which application of conventional methods is infeasible. Of the 4 methods of in vitro preservation of germlasm, viz., cryopreservation, slow growth shoot culture, normal growth culture and regenerative excised root culture, the last method has some advantages over the others in case of certain plant species, particularly the palms. Its main advantages are: Practicability and low cost because of no requirement of agar-agar, of light as well as of strict maintenance of temperature besides easy exchange of germlasm across the International boundaries without damage in transit. However, all these 4 methods have certain shortcomings, which necessitate adoption of a composite approach involving all of them to achieve the main goal of conservation of phytodiversity. In general, durations of germlasm preservation in vitro through cryopreservation and slow growth shoot culture have ranged from a few weeks to about 2 yrs depending on the plant species concerned. In certain plant species, the normal growth culture had afforded germlasm preservation for considerably longer periods of time, viz., more than 27 yrs in Dioscorea floribunda and D. deltoidea and 32 yrs in Citrus grandis, albeit with a danger of cultures getting infected during frequent subcultures. In contrast, the duration of germlasm preservation through regenerative excised root culture ranged from 6 to 24 yrs. The method of regenerative excised root culture had been demonstrated to preserve germlasm of a number of plant species, including herbaceous annuals and perennials and trees, viz., Solanum khasianum (spiny and spineless), S. torum, S. surattense, Atropa belladonna, Kalanchoe fedtschenkoi, Rauwolfia serpentina, Populus deltoides and Dalbergia latifolia. A similar possibility existed in case of Shorea robusta, Cocos nucifera and Elaeis guineensis, roots of which though established in long-term culture, the regenerant differentiation in their explants was most sporadic. For establishing excised root cultures of different plant species, modifications of various nutrient media, viz., Murashige and Skoog (1954), Street and McGregor (1952) and White (1943) were used in liquid state, while for inducing regenerant differentiation, some other modifications of the same media except of White as well as of Schenk and Hildebrandt (1972) medium were employed, using different cytokinins, viz., BAP, 2iP, Z and TDZ with auxins, viz., IAA, NAA and 2, 4-D and also certain growth inhibitors/retardants, viz., ABA, CCC and ancymidol as also a polyamine, putrescine. In all cases, agarified media were used except in R. serpentina, which required the liquid state of medium for regenerant differentiation, while in S. khasianum and A. belladonna, caulogenesis took place in agarified morphogenic medium and embryogenesis in the liquid state of medium. Further, whilst in R. serpentina and A. belladonna plantlets were produced via embryogenesis, in rest of the plant species through caulogenesis. Plants regenerated through long-term excised root culture were true-type, which was substantiated by tracing their origin from the pericycle tissue of root explants. The root-regenerated plants fared well under field conditions save A. belladonna, plants of which could be grown in potted soil only in controlled physical conditions.

Keywords: Caulogenesis, conservation, embryogenesis, excised root culture, germlasm preservation, phytodiversity, regenerants from pericycle, root-regenerated plants

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Introduction

Conservation of germlasm of domesticated and wild plant species is essential for survival of mankind on this planet. Besides wanton extraction of plants, regional climatic changes are the main cause of vanishing genotypes as also species and genera. It is reported that many of the plant species might have become extinct before being evaluated for their precious role in human welfare. Such a situation is documented in Red Data Books (1987, ’88 and ’90) of Botanical Survey of India. Whilst resurrection of extinct germlasm is not possible, the endangered genotypes can be preserved and the phytodiversity conserved in situ.

A prior assessment is warranted to decide as to which strategy has to be adopted for germlasm pres-
ervation of different plant species varying in genetic make-up, regeneration nature and potential and their absolute requirement of specific agro-climates for survival. Thus, the conventional methods, like, Field Gene Banks and Seed Banks may be inadequate for preservation of germplasm of certain plant species. In such situations, employment of in vitro strategies for germplasm preservation becomes imperative. For example, in case of heterozygous plant species, which must be propagated vegetatively or those where seeds are not produced or those found in unmanageable habitats or which are systemically infected or are available in very small quantities, viz., transgenics.

In vitro germplasm preservation is possible through different means, prominent amongst which are: (i) cryopreservation with its different facets, (ii) slow growth shoot culture employing low temperature and light intensity as well as using balanced amounts of growth promoter/s and inhibitor/s in the culture media, (iii) normal growth shoot culture and (iv) regenerative excised root culture. The first and second aspects along with related biotechnological considerations are covered in some recent books and reviews. In any approach, the main considerations have to be genetic stability of the preserved germplasm in long-term duration, its substantially enough regeneration capacity after preservation and practicability of the process. Although there are a few instances of cryopreservation of certain plants, particularly of those possessing cold hardiness trait, the short duration of preservation and generally a very poor retrieval of potentiality during long-term culture, which is inherent in many plant species.

In certain cases, shoot apices cannot be cultured either due to their scarcity or unmanageable size as in palms, which amounts to discredit to the processes of slow growth shoot culture as also of cryopreservation. In this context, the long-term regenerative excised root culture is innovative and outstanding, particularly for the long durations for which the germplasm of varied plant species had been preserved ranging from 13-24 yrs. The disadvantages pointed out for cryopreservation as well as for slow growth shoot culture are circumvented in this process besides several other advantages, main amongst which are: Normal growth of preserved explant, simple culture conditions, i.e., no requirement of agar-agar, of light as also of strict maintenance of low temperature and economy of space due to accommodation of metres long roots in small containers having the potential to produce enormous number of propagules per culture, all of which lead to low maintenance cost. Besides practicability and low cost, such attributes give this system an edge over the other 2 systems in promoting safe exchange of germplasm over long distances across the International boundaries unaffected by vagaries of transit period, mainly temperature fluctuations and lack of light.

There are reports of regeneration from excised root culture of Brassica oleracea and C. aurantifolia. However, both the reports lack information so as to qualify for the system of germplasm preservation through long-term excised root culture. While the potentiality of excised root culture in B. oleracea to regenerate shoots was lost beyond five months, the regeneration in explants from excised root cultures of C. aurantifolia was very sporadic and scanty and only the segments with tip responded to regenerate a solitary shoot bud. Unless segments from the whole length of cultured excised root have the regeneration potentiality, the extent of length of root in culture will be superfluous and cannot qualify for establishment of a germplasm preservation system—a Gene Bank.

On the other hand, there are several plant species, root segments of which have been reported to regenerate, indicating a possibility of developing regenerative excised root culture for their germplasm preservation, viz., Camaenerion angustifolium, Isatis
tinctoria\textsuperscript{15}, Convolvulus arvensis\textsuperscript{16,17}, Elaeis guineensis\textsuperscript{18}, \textit{Actinidia chinensis}\textsuperscript{19}, Phalaenopsis amabilis\textsuperscript{20}, Populus sp.\textsuperscript{21}, \textit{C. sinensis}\textsuperscript{22,23}, \textit{Cichorium intybus}\textsuperscript{24}, \textit{Catasetum trullu}\textsuperscript{25}, \textit{B. napus}\textsuperscript{26}, \textit{Solanum tuberosum}\textsuperscript{27}, \textit{Vanda} hybrid (\textit{Vanda TMA X V. teres}) and \textit{Rhyynchostylis retusa}\textsuperscript{28}, \textit{Lotus coriiculatus}\textsuperscript{29}, \textit{Dendrobium}\textsuperscript{30}, \textit{P. nigra}\textsuperscript{31}, \textit{P. tremula}\textsuperscript{32-34}, \textit{tomato}\textsuperscript{35}, \textit{Cephaelis ipecacuanha}\textsuperscript{36}, \textit{Acacia albida}\textsuperscript{37} and \textit{Albizia julibrissin}\textsuperscript{38}.

The first report on regenerative excised root culture, which could be employed for developing a process of germplasm preservation, as a Gene Bank had been on \textit{Solanum khasianum}\textsuperscript{39}. Subsequently, some other plant species were also demonstrated to provide regenerative excised root cultures beyond annuals and herbaceous plant species leading to perennials and trees\textsuperscript{11, 40-42}. Amongst trees, regenerative excised root cultures: \textit{R. serpentina} and \textit{P. deltoides} and \textit{Street and McGregor} medium\textsuperscript{45} for \textit{S. torvum}, \textit{S. surattense} and \textit{A. belladonna} and Murashige and Skoog medium (MS)\textsuperscript{46} for rest of the plant species. In certain cases, excised roots were initially established in a particular treatment, but for long-term culture modifications of a totally diverse medium, were used, viz., modifications of \textit{Street} and \textit{McGregor} medium were used for initiating root cultures of \textit{S. torvum} and \textit{A. belladonna}, but for long-term culture they had to be maintained in the modifications of \textit{White} medium\textsuperscript{47} and MS medium, respectively. Similarly, the excised root cultures of \textit{P. deltoides}, while initiated in a modification of \textit{Street} medium periodically required deletion of NOA for continued growth of excised roots. On the contrary, the excised roots of \textit{D. latifolia} for their long-term culture were grown in the same medium in which they were established, but with the addition of KNO$_3$ and m-inositol after 8 yrs of initiation of cultures.

For differentiation of regenerants, 1-cm-long root segments were obtained from well-differentiated portion of excised roots, while the tip portion (where the maturation of differentiating tissue was yet to take place) was discarded. For most of the plant species, modifications of the MS medium were used, while in some cases modifications of a few other media had to be employed, viz., Schenk and Hildebrandt medium (SH)\textsuperscript{48} for \textit{D. latifolia} and \textit{A. belladonna} and \textit{Street} medium for \textit{R. serpentina}. Also, while in most of the cases the agarified medium was used, in \textit{R. serpentina} the liquid state of the medium was employed and in still other cases, like, \textit{S. khasianum} and \textit{A. belladonna} both agarified and liquid media were used. In majority of plant species, BAP along with an auxin was used at a particular ratio, but in some instances 2iP, Z as also TDZ were additionally incorporated both individually and in certain combinations. In certain other cases, along with cytokinin/s, ABA or CCC or ancymidol was employed, save the exceptional situation where it was not possible to have shoot apices of the two palms, while shoots taken from mature tree of \textit{S. robusta} did not root. Alternatively, the leaf segments of \textit{C. nucifera} were induced to produce roots, which comprised the explants for establishing root cultures.

Whilst giving an account of all the details of various modifications made in different liquid nutrient media and the treatments employed for establishing root cultures of different plant species investigated is beyond the scope of this paper, suffice it to mention that \textit{Street} medium\textsuperscript{44} was modified for root cultures of \textit{R. serpentina} and \textit{P. deltoides} and \textit{Street} and \textit{McGregor} medium\textsuperscript{45} for \textit{S. torvum}, \textit{S. surattense} and \textit{A. belladonna} and Murashige and Skoog medium (MS)\textsuperscript{46} for rest of the plant species. In certain cases, excised roots were initially established in a particular treatment, but for long-term culture modifications of a totally diverse medium, were used, viz., modifications of \textit{Street} and \textit{McGregor} medium were used for initiating root cultures of \textit{S. torvum} and \textit{A. belladonna}, but for long-term culture they had to be maintained in the modifications of \textit{White} medium\textsuperscript{47} and MS medium, respectively. Similarly, the excised root cultures of \textit{P. deltoides}, while initiated in a modification of \textit{Street} medium periodically required deletion of NOA for continued growth of excised roots. On the contrary, the excised roots of \textit{D. latifolia} for their long-term culture were grown in the same medium in which they were established, but with the addition of KNO$_3$ and m-inositol after 8 yrs of initiation of cultures.

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putrescine was used. In case of auxins, besides IAA and NAA, 2, 4-D was also used, particularly in palms.

The isolated developed shoots, which had proper tissue differentiation, measuring 3-5 cm in length, depending on the plant species, were subjected to root inducing treatments. In all the cases, modifications of MS medium effected especially in respect of concentrations of inorganic salts were applied except in case of A. belladonna where a modification of SH medium was used. And amongst all the plant species tested, shoots only of D. latifolia required a 2-step procedure for root induction. Furthermore, while in most of the plant species an agarified rooting medium was used, in case of P. deltoides only the liquid medium with filter paper bridge was employed and in D. latifolia, culture of shoots initially in agarified medium was followed by their subculture in liquid medium. Of the various auxins used, IAA was the auxin of choice in most of the plant species except a few, where other auxins were used, viz., NAA or indole-3-butyric acid (IBA). A special mention needs to be made regarding rooting in isolated shoots of A. belladonna and D. latifolia. In the former case, along with a combination of 0.1 mg l⁻¹ of IBA and NOA, 0.5 mg l⁻¹ each of phloroglucinol and catechol were used, while in the latter, isolated shoots were incubated first in 0.25 mg l⁻¹ IAA in an agarified medium followed by their transfer to an auxin-free liquid medium with filter paper bridge. Mostly, auxins whether used alone or in combination with another auxin, their concentrations ranged from 0.1-0.5 mg l⁻¹. Initially all the rooting cultures were incubated in dark for 2 to 3 days.

The pH of all the media was adjusted, before autoclaving, to 4.9-6 depending on the plant species and they were sterilized by autoclaving at 1.08 kg/cm² for 15 min. Whilst the root cultures were kept in dark, rest of the cultures were maintained under 25 μmol m⁻² s⁻¹ photon flux density of light for 15 hrs a day and all of them were incubated at 27°C ± 1°C with 70 ± 5% RH maintained in the culture room.

The plantlets, both raised from somatic embryos and shoot buds, were nurtured to a size of 6-8 cm in length before hardening them for transplantation to soil following the procedure developed for D. floribunda. Also, histological preparations were made for tracing the origin of differentiation of regenerants.

Results and Discussion

The various optimum treatments (nutrient media) derived for establishment and growth of excised roots, differentiation of regenerants in root explants, their proliferation and ultimately formation of plantlets of different plant species investigated are given in Table 1.

Long-term regenerative excised root cultures of S. khasianum, spiny and spineless, 24 and 20 yrs of age, respectively, served as the test cases. Germplasm of S. khasianum spineless, being annual and a cross-pollinated hybrid could not be maintained through seeds nor through vegetative means, i.e., by cuttings. Its excised roots grew normally during the course of 20 yrs, observed so far, but interestingly enough, requirement of vitamins for its excised root growth was qualitatively far more than its spiny parent. The excised roots of both spiny and spineless S. khasianum had the same appearance and rate of growth in culture (Fig. 1). However, the shoots regenerated from root segments showed in vitro their characteristic traits of being spiny or spineless from the beginning (Fig. 2), while the root-regenerated plants of spineless genotype did not show any spines up to the last stage of development, i.e., fruiting (Fig. 3). All the regenerated plants from long-term cultures of excised roots were true-to-type in their morphological characters. It was further substantiated by tracing the origin of differentiation of regenerants in root explants, which was invariably from the pericycle tissue (Fig. 4), which represented the exact genetic make-up of the mother plant. There was no change in the requirement of growth hormones nor in the magnitude of differentiation of regenerants in explants taken from initial or long-term root cultures. In both the cases, the root-regenerated plants developed normally under field conditions and produced berries at par with plants raised from seeds, albeit seeds from spineless genotype produced plants with all grades of spiny trait.

Excised root cultures of S. torvum and S. surattense responded to the BAP and NAA containing media after the manner of S. khasianum, but the magnitude of differentiation was slightly more. The root-regenerated plants survived 100% after transplantation and within 4 months of transplantation to field fruited profusely. However, the difference in the morphogenetic pattern of differentiation was obvious between these 2 species and S. khasianum in that while the root explants of the latter differentiated well-formed embryos in the liquid state of the same morphogenetic medium, explants of the former 2 species did not differentiate any regenerants in the liquid state of the medium. The phenomenon of the shift in
Table 1—Optimum treatments for different stages involved in germplasm preservation through regenerative long-term excised root cultures of certain plants

<table>
<thead>
<tr>
<th>Stages</th>
<th>Optimum treatments * for respective plants and kind of regenerant differentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. khasianum</strong> (spiny)</td>
<td></td>
</tr>
<tr>
<td>1. Establishment of excised root culture</td>
<td>Mod MS medium Thiamine-HCl 0.1, Pyridoxine-HCl 0.1, Nicotinic acid 0.0, Folic</td>
</tr>
<tr>
<td></td>
<td>acid 0.1, d-Biotin 0.1, Riboflavin 0.1, Sucrose 4% &amp; pH 5.2</td>
</tr>
<tr>
<td>2. Root growth in long-term culture</td>
<td>Mod White medium d-Biotin 0.1 &amp; l-Arginine-HCl 5</td>
</tr>
<tr>
<td>(Age of culture)</td>
<td>(24 Yrs)</td>
</tr>
<tr>
<td>3. Regenerant differentiation in root segments</td>
<td>Mod MS medium BAP 0.25, NAA 0.01 &amp; AdS 5</td>
</tr>
<tr>
<td>i) Kind of regenerant</td>
<td>Sh buds in agar medium</td>
</tr>
<tr>
<td>ii) Incubation period</td>
<td>10–12 Days</td>
</tr>
<tr>
<td>4. Proliferation of shoots/ embryoids</td>
<td>Mod MS medium BAP 0.25, NAA 0.01 &amp; AdS 5</td>
</tr>
<tr>
<td>5. Rooting of isolated shoots (plantlets)</td>
<td>Mod MS medium IAA 0.1 &amp; AdS 5</td>
</tr>
</tbody>
</table>

| **S. khasianum** (spineless)                    |                                                                                 |
| 1. Establishment of excised root culture        |                                                                                 |
| 2. Root growth in long-term culture             |                                                                                 |
| 3. Regenerant differentiation in root segments  |                                                                                 |

| **S. torvum**                                  |                                                                                 |
| 1. Establishment of excised root culture        |                                                                                 |
| 2. Root growth in long-term culture             |                                                                                 |
| 3. Regenerant differentiation in root segments  |                                                                                 |

| **S. surattense**                              |                                                                                 |
| 1. Establishment of excised root culture        |                                                                                 |
| 2. Root growth in long-term culture             |                                                                                 |
| 3. Regenerant differentiation in root segments  |                                                                                 |

*Note: MS medium contains: Thiamine-HCl 0.1, Pyridoxine-HCl 0.1, Nicotinic acid 0.1, Folic acid 0.1, d-Biotin 0.1, Riboflavin 0.1, Sucrose 4% & pH 5.2. Mod MS medium is modified MS medium. Mod Street medium is modified Street medium. Mod McGregor medium is modified McGregor medium. ABA is abscisic acid. NAA is naphthalene acetic acid. BAP is benzyl adenine. NAA is naphthalene acetic acid. AdS is adenine. ME is methyl cellulose. IAA is indole acetic acid. NOA is nicotinic acid. Z is Zn. Putrescine is putrescine.

(Contd.)
**Table 1—Optimum treatments for different stages involved in germplasm preservation through regenerative long-term excised root cultures of certain plants—Contd.**

<table>
<thead>
<tr>
<th>Stages</th>
<th>S. khasianum (spiny)</th>
<th>S. khasianum (spineless)</th>
<th>S. torvum</th>
<th>S. surattense</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) Kind of regenerant</td>
<td>Sh buds in agar medium</td>
<td>Sh buds</td>
<td>Embs</td>
<td>Sh buds</td>
</tr>
</tbody>
</table>
| 4. Proliferation of shoots/embryoids | Mod SH medium
BAP 0.05, IAA 0.25,
AdS 15 & CCC 5 | Mod MS medium
BAP 0.25, NAA 0.1 & AdS 50 | Mod SH medium
BAP 0.5, IAA 1 & AdS 15 | Ibid |
| 5. Rooting of isolated shoots (plantlets) | Mod SH medium
IBA 0.1, NOA 0.1, Phloroglucinol 0.1 & Catechol 0.1 | Mod MS medium
NAA 0.1 | Mod MS medium
NAA 0.5 | Mod MS medium
IAA 0.25 |
| 1. Establishment of excised root culture | Mod MS medium
Thiamine-HCl 0.1, Pyridoxine-HCl 0.1, Nicotinic acid 0.1, Folic acid 0.1, d-Biotin 0.1, Riboflavin 0.1, NAA 0.01 & pH 5.2 | Mod MS medium
Cysteine-HCl 25, Ascorbic acid 15, IAA 0.05 & pH 5.2 | Mod MS medium
l-Glutamine 50, Riboflavin 0.01, m-Inositol 20, Sorbitol 10, Ancymidol 0.01, NOA 0.01 & pH 5.2, Mod Street medium
Ca (NO₃)₂ 200, Na₂SO₄ 100, NOA 0.01 & pH 5.2 |
KNO₃ 100 & m-Inositol 20 | Mod MS medium
(NH₄)₂SO₄ 100, Nicotinic acid 0.5, d-Biotin 0.1 & NAA 0.05 | Ibid
l-Glutamine 50, Riboflavin 0.01, m-Inositol 20, Sorbitol 10, Ancymidol 0.01, NOA 0.01 & pH 5.2 |
| 3. Regenerant differentiation in root segments | 3-Step Process
Mod SH medium
BAP 0.25, IAA 0.025, AdS 15 & CCC 15 | 3-Step Process
Mod MS medium
BAP 1, IAA 0.25 & AdS 15 | 3-Step Process
Mod MS medium (with activated charcoal 2g/l)
i) 2,4-D 70, NAA 20 & Z 5
ii) 2,4-D 70, NAA 20 & BAP 10
iii) 2,4-D 25 & BAP 15
i) 2,4-D 70, NAA 20, BAP 10 & CH 200
ii) 2,4-D 25, BAP 5, 2 iP 5, TDZ 10 & AdS 50 | 2-Step Process
Mod MS medium
BAP 0.25, IAA 0.025, AdS 15 & l-Glutamine 75 | 2-Step Process
Mod MS medium (with activated charcoal 3g/l)
i) 2,4-D 70, NAA 20, BAP 10 & CH 200
ii) 2,4-D 25, BAP 5, 2 iP 5, TDZ 10 & AdS 50 |
| i) Kind of regenerant | Sh buds | Meristematic globular bodies | Meristematic globular bodies at the sites of lateral roots | Embs (most sporadic) |
| ii) Incubation period | 75-80 Days | 30-40 Days | 100-120 Days | 90-100 Days |
| 4. Proliferation of shoots/embryoids | Mod SH medium
BAP 0.25, IAA 0.025, AdS 15, CCC 15 & l-Glutamine 75 | — | — | — |
| 5. Rooting of isolated shoots (plantlets) | 2-Step Process
Mod MS medium
i) IAA 0.25 (agar medium)
ii) BM (liquid medium with filter paper bridge) | — | — | — |

* Concentrations in mg l⁻¹
* Mod, modified; Sh, shoot; Embs, embryos
the morphogenic pattern of differentiation from caulogenesis to embryogenesis, with change in the state of the medium from semisolid to liquid, is of great practical advantage. Embryogenesis is preferred to shoot bud differentiation for the sake of mass production of embryos in bioreactors with a readymade root system and being a complete entity they are suitable to be used for developing synthetic seeds to facilitate in vitro storage and mechanical sowing. The phenomenon acquires more significance in case of trees, where shoots with adventitious roots may prove greatly disadvantageous as compared to that having a single tap root-like system, which strongly anchors them with soil. Such a shift of morphogenic pattern of differentiation is similar to that reported in *C. grandis* except that it was the function of the stem callus tissue, which became habituated and the change from caulogenesis to embryogenesis was effected by a change in the cytokinin used from BAP to Z.\(^{51}\)

In *A. belladonna*, the pattern of regenerant differentiation in root explants was still closer to *S. khasianum*, but there was a far more propensity of embryogenesis in a liquid morphogenic medium, which was augmented in the presence of 0.001 mg l\(^{-1}\) ABA (Fig. 5). The role of ABA appeared to not only augment embryogenesis, but to help promote synchronized development and maturation of embryos after the manner of *Carum carvi\(^{52}\). Regeneration of normal plants from excised roots cultured for more than 18 yrs substantiates a major attribute of germplasm preservation of plants requiring extremely different agro-climates for their growth than the place where the germplasm is preserved, as *A. belladonna* cannot possibly be grown under Lucknow agro-climate. Root-differentiated embryos produced normal healthy plants of *A. belladonna* under culture room conditions. Similarly, a profuse number of shoots were obtained from a few shoot buds differentiated with the conversion of root meristem into a shoot meristem in segments of about 13-yr-old excised root cultures of *K. fedtschenkoi*.

Of special relevance is the preservation of germplasm of *R. serpentina*, a miracle drug plant of India, which is highly endangered. This is another important attribute of the in vitro conservation strategy, in which excised roots of more than 20 yrs of age in culture differentiated embryoids, which on germination produced crops of proliferating shoots for production of enormous number of plants for replenishment of its depleted habitats (Fig. 6). Besides BAP and Z used with NOA, presence of particularly putrescine alone with ABA and m-inositol triggered differentiation of embryoids in liquid medium. Role of polyamines in somatic embryogenesis is now well-established especially in association with ethylene biosynthesis, as well demonstrated in cultures of nucellar tissue of mango\(^{53}\). However, the exact mode of action of polyamines in inducing somatic embryogenesis in tissues, which are not programmed to produce somatic embryos in contrast with the nucellar tissue of polyembryonic varieties, is not yet clear. Similarly, the conducive role played by m-inositol in somatic embryogenesis needs to be investigated, if it is due to its additive effect as a cytokinin along with potent cytokinins, i.e., BAP and Z.

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**Figs 1-4**—*Solanum khasianum* (spineless) : 1, A long-term (20 yrs) culture of excised roots. Bar = 1 cm; 2, Regeneration of shoots from root segments taken from excised root culture. Bar = 1 cm; 3, A fruiting twig of field-grown true-to-type plant regenerated through excised root culture showing no spines. Bar = 2 cm; 4, Differentiation of a shoot meristem from pericycle tissue of a root explant. Bar = 0.1 mm; Fig. 5, Multiple embryos of different shapes and sizes differentiated from long-term (18 yrs) excised root culture of *Atropa belladonna*. Bar = 1 cm & Fig. 6, Field cultivation of *Rauvolfia serpentina* plants regenerated from its excised root culture. Bar = 10 cm
Germplasm preservation of trees is far more arduous and challenging than of herbaceous plants, mainly because of the slow growth, long life span and huge size, if not also the intractable-to-regenerate nature. In general, excised root cultures of trees is comparatively very intractable than those of herbaceous plants. In this background, germplasm preservation of 4 clones of *P. deltoides*, viz., G3, G48, S7C15 and D121 and of *D. latifolia* assumes special significance. While in the former case the main difficulty had been to achieve sustained growth of excised roots in long-term culture, in the latter the propensity of callusing of explants taken from long-term excised root cultures had been a difficult problem to be alleviated. In *P. deltoides*, incorporation of 0.01 mg l$^{-1}$ NOA in the presence of m-inositol gave an initial boost to formation of laterals, which along with the main root also grew. However, the root growth progressively declined after a few subcultures, necessitating withdrawal of NOA, which had to be incorporated again in the medium for formation of laterals and root growth. Of the four clones of *P. deltoides*, better growth of excised roots was obtained in S7C15, albeit potentiality of shoot bud differentiation was same in all the four clones. Explants taken from ca. 10-yr-old excised root cultures of *P. deltoides* clone G3 (Fig. 7), differentiated multiple shoot buds from their pericycle tissue on an agarified medium containing BAP and IAA within a period of 20-25 days, which produced normal-looking plants in potted soil after another about 60 days (Figs 8 to 10).

Excised roots of *D. latifolia* continued to grow in long-term culture, registering about 20-fold growth in 60 days. As mentioned earlier, segments taken from excised roots had a propensity to callus on morphogenic medium. Callusing of root explants in the minimum effective concentrations of BAP and IAA for regenerant differentiation, i.e., 0.25 mg l$^{-1}$ and 0.025 mg l$^{-1}$, respectively, could be contained to a great extent with the incorporation of growth retardants, viz., CCC or ancymidol along with the reduction in the concentration of BAP from 0.25 to 0.1 mg l$^{-1}$ in the medium used for subsequent subcultures. Differentiation of shoot buds took about 75 to 80 days. Once a few shoot buds were formed, they gave rise to cultures of proliferating shoots. The isolated shoots rooted in a 2-step procedure, in which root primordia differentiated in an agarified rooting medium under the influence of 0.25 mg l$^{-1}$ IAA, while the root growth took place with minimal intervening callusing in an auxin-free liquid medium resulting in plantlets (Fig. 11).

By judiciously following the hardening procedure, the transplant success achieved was 100% in *S. khasianum* (spiny and spineless), *S. torvum*, *S. surattense*, *K. fedtschenkoi* and *R. serpentina* and about 90% in *P. deltoides* and *A. belladonna*. It took about 3 months to obtain a root-regenerated potted plant of a herbaceous plant species and about 5 months in case of *P. deltoides* from the time of regenerant differentiation in root explants.

*S. robusta*, a source of toughest wood unaffected by termites and most resistant to withering, is equally
difficult to be regenerated. The intractable-to-
regenerate nature was also reflected in its excised root
culture. Excised roots had been growing satisfac-
torily, registering about 30-fold increase in length with
laterals included, during 60 days’ incubation, for 21
years, observed so far. However, their explants eluded
regenerant differentiatiation in any of the several mor-
phogenic treatments tested, except that some meris-
tematic globular structures were formed indicating the
possibility of regenerant differentiation.

As yet, no method is available for germplasm pres-
servation of palms in the true sense, while there exists
much genetic variation in them. Particularly, *C. nucifera*,
which takes about 15 years to come to fruiting stage,
is a complex hybrid with inherent great genetic
variation, has solitary shoot meristem and cannot be
vegetatively propagated. In such cases, development
of *in vitro* methods for cloning of mother trees and
preservation of their germplasm is invaluable. Long-
term regenerative excised root cultures of *C. nucifera*
as also of *E. guineensis* hold a great promise to this
end. Initial success obtained in establishing their ex-
cised root cultures had been encouraging (Figs 12 &
13). More so, when somatic embryos were differenti-
ated, albeit most sporadically in root explants of *E.
guineensis* and meristematic globular bodies differenti-
tiated at the sites of emergence of lateral roots along
the length of excised roots of *C. nucifera* (Figs 14 &
15). In both the cases, root explants subjected to a
pulse treatment, an exceptionally high concentration
of 2, 4-D, i.e., 70 mg l⁻¹ along with 20 mg l⁻¹ NAA
and 5 mg l⁻¹ Z in case of *C. nucifera* and 20 mg l⁻¹
NAA and 10 mg l⁻¹ BAP in case of *E. guineensis* fol-
lowed by their subculture in moderately high concen-
trations of 2, 4-D and BAP in case of the former and
2, 4-D along with BAP, 2iP and TDZ in the latter re-
resulted in regenerant differentiatiation.

Like any other process, the regenerative excised root
cultures may not be considered as a panacea for
germplasm preservation of all kinds of plant species,
since excised roots of all the plant species may not be
amenable to culture and if established in culture it is
an altogether different proposition to induce regener-
ant differentiation in them, particularly in long-term
culture. For example, bulbous and tuberous crops, the
whole group of cacti, halophytes as also mangroves
may be most intractable, if not impossible, to be es-
ablished in terms of their regenerative excised root
cultures. It is analogous to the situation, where all the
plant species cannot be propagated by root cuttings,
stem cuttings or leaf cuttings. Thus, a composite ap-
proach will be to involve all the strategies of germ-
plasm preservation, depending on the availability of
the most regenerative plant part in a particular plant
species for *in vitro* culture.

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