In vitro high frequency regeneration of plantlets of Vigna mungo and their ex vitro growth

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Received 13 June 2000; revised 5 May 2001

Of the five explants of V. mungo var. T9 used, the excised shoot tips gave best response with regard to offshoot formation followed by the embryonal axis explants. While a treatment comprising 0.5 mgL⁻¹ BAP, 0.5 mgL⁻¹ 2iP and 0.1 mgL⁻¹ NAA induced differentiation of an average 10 offshoots in shoot tip explants, only 3 offshoots were formed in the explants of embryonal axis in a treatment containing 0.5 mgL⁻¹ BAP and 0.1 mgL⁻¹ NAA, found optimum for them. Multiple shoots differentiated when explants with earlier regenerated and growing offshoots were first cultured in a treatment containing 0.1 mgL⁻¹ BAP, 0.25 mgL⁻¹ IAA and 5 mgL⁻¹ CCC and then subcultured in the same treatment but having only 1 mgL⁻¹ CCC. The isolated shoots rooted in 0.5 mgL⁻¹ IAA resulted in the formation of complete plantlets of an average height of 15 cm in 20 days. The in vitro-regenerated plants grew normally under field conditions and came to flowering as well.

Legumes as a group have been considered intractable to regenerate in vitro. However, recently a number of successful regeneration protocols have been developed for grain legumes, specially with a view to facilitate their genetic transformation¹, where most of the regeneration studies have been concerned with the cotyledonal explants²⁻⁴. However, differentiation from a mature storage organ, like, cotyledon may not be preferred for clonal multiplication as it has been reported to generally consist of genetically variable cells⁵⁻⁷. In another phase of regeneration studies in legumes, the embryonal axis has been the explant of choice, so also in Vigna mungo, besides the cotyledonal explants⁸. In embryonal axis explants of V. mungo not only has a low number of offshoot formation been reported, but an intervening callusing phase has been associated with offshoot regeneration⁹. The present communication reports on morphogenetic studies carried out with different vegetative explants of V. mungo for assessment of their relative regeneration potential, which was found highest in excised shoot tip culture. The regenerated shoots were rooted and raised to complete plants, which came to reproductive phase under field conditions. De novo differentiation of meristems of offshoots as obtained in excised shoot tip culture and its proliferation will make it a preferred method to be used for genetic transformation studies as compared to culture of other kinds of explants.

Materials and Methods

Seeds of Vigna mungo L. variety T9 were pre-treated with Teepol (Qualigen) solution (5%) for 5 min, washed with single distilled water, surface-sterilized with 0.2% aqueous solution of HgCl₂ for 5 min and finally thoroughly rinsed with sterile distilled water to remove all traces of HgCl₂. The surface-sterilized seeds were soaked for 2 hr in sterile distilled water and inoculated, one seed in each culture tube, on White’s⁹ and Murashige and Skoog’s¹⁰ (MS) media solidified with 0.75% agar. In both media, 5 mgL⁻¹ adenine sulphate (AdS) was incorporated and pH of all the media was adjusted to 5.8 before adding agar and they were sterilized by autoclaving at 1.08 kg/cm² for 15 min. Cultures were incubated under 37.5 μmol m⁻²S⁻¹ fluorescent light for 15 hr photoperiod at 27° ± 1°C. Various explants, namely, shoot tips, segments of leaf, hypocotyl, embryonal axis and radicle close to hypocotyl, were excised from 7-day-old aseptically-grown seedlings and inoculated in liquid medium on filter paper bridge as well as on agarified nutrient medium of same composition.

For morphogenetic studies, a modified MS medium was used. In this medium, the NH₄NO₃ concentration was reduced from 1650 mgL⁻¹ to 500 mgL⁻¹, that of KNO₃ from 1900 mgL⁻¹ to 1000 mgL⁻¹, meso-inositol
was omitted and L-glutamine was added from 50-100 mgL⁻¹. Effect of different cytokinins, namely, 6-benzylaminopurine (BAP), N⁶-(2-isopentenyl) adenine (2iP) or kinetin (Kn) used at different concentrations and in combinations with indole-3-acetic acid (IAA) or α-naphthaleneacetic acid (NAA) were studied on induction of offshoot regeneration. For further proliferation and growth of regenerated shoots, effect of 1, 2.5, 5 and 7.5 mgL⁻¹ 2-chloroethyltrimethyl-ammonium chloride (CCC) was used in 1: 3 ratio. For ex vitro growth of plantlets, compost (farm-yard) and garden soil were used in 1: 3 ratio.

Results and Discussion

Results of preliminary experiments using only embryonal axis as explant, showed NAA as a more potent auxin than IAA at any of its several concentrations tested with different concentrations of BAP for induction of offshoots. Similarly, BAP was more effective than Kn in this respect. A combination of 2iP and BAP along with NAA was more effective than the treatment where Kn was substituted for 2iP. Although preference for a particular auxin appears to be species specific or even explant specific, it is generally the synthetic auxins, which have been reported to be more effective in comparison to natural ones as in the present case. The same is true with cytokinins where 2iP, though a natural cytokinin has been found less effective than BAP. In case of Citrus species, BAP has generally been found most effective, but Bouzid has reported regeneration of shoot buds in stem explants of C. aurantium, C. reticulata and C. limon by Kn. But it is contrary to the general experience that different explants of the same plant species preferred different cytokinins for offshoot formation as in the present case; Kn being more efficacious for shoot tip explants, while BAP for those of embryonal axis.

In general, however, Kn appeared to be more effective in shoot tip explants in subsequent experiments, which were conducted with all the five kinds of explants, namely, shoot tips and segments of leaf, hypocotyl and radicle besides embryonal axis. All the explants employed for induction of offshoot differentiation, the best response was obtained with shoot apices followed by embryonal axis, while rest of the explants mostly calloused or remained quiescent. Similarly, most of the treatments comprising individual auxins or cytokinins or their different combinations did not evoke morphogenetic response in most of the explants, a full account of which is beyond the scope of the present paper. In Table 1 are presented the responses obtained in respect of shoot apices and embryonal axes in some of the selected treatments in order to identify the optimum treatments for the two responsive explants.

Shoot tip explants in the optimum treatment comprising 0.5 mgL⁻¹ each of BAP and 2iP along with 0.1 mgL⁻¹ NAA, produced on an average more than 10 offshoots within 20 days, while from the embryonal axis explant only 3 offshoots per explant were formed in the same treatment. Synergism between two auxins with reference to rooting effect is well-documented in literature. But regarding a similar synergism existing between cytokinins, where a combination of two cytokinins becomes far more effective than either of the cytokinins, there are some reports also coming up with in vitro culture of different plants species. An increase in efficacy of a cytokinin when combined with another cytokinin mainly of a combination of BAP and 2iP has been reported in cultures of some other plants species.

On the contrary to the treatment containing both BAP and 2iP, a maximum number of 4 offshoots were produced per embryonal axis explant in a treatment comprising 0.5 mgL⁻¹ BAP and 0.1 mgL⁻¹ NAA, while only about 3 offshoots were produced in the

<table>
<thead>
<tr>
<th>Growth hormones</th>
<th>Time taken (days)</th>
<th>No. of shoots/explants</th>
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<tbody>
<tr>
<td>NAA 0.25</td>
<td>25</td>
<td>3.6 ± 0.68</td>
</tr>
<tr>
<td>BAP 0.5 + NAA 0.25</td>
<td>25</td>
<td>1.4 ± 0.25</td>
</tr>
<tr>
<td>BAP 0.5 + NAA 0.1</td>
<td>20</td>
<td>3.2 ± 0.37</td>
</tr>
<tr>
<td>BAP 1.0 + NAA 0.1</td>
<td>25</td>
<td>1.8 ± 0.38</td>
</tr>
<tr>
<td>Kn 1.0 + NAA 0.1</td>
<td>20</td>
<td>5.83 ± 0.52</td>
</tr>
<tr>
<td>BAP 0.5 + 2iP 0.5 + NAA 0.1</td>
<td>20</td>
<td>10.2 ± 1.5</td>
</tr>
<tr>
<td>BAP 0.5 + Kn 0.5 + NAA 0.1</td>
<td>30</td>
<td>2.0 ± 0.45</td>
</tr>
</tbody>
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corresponding shoot tip explants during a period of 20 days. In a concentration higher than 0.5 mgL\(^{-1}\) BAP, i.e., 1 mgL\(^{-1}\), only callus was formed in embryonal axis explants which response was similar in case the concentration of NAA was increased from 0.1 mgL\(^{-1}\) to 0.25 mgL\(^{-1}\) even when the concentration of BAP was maintained as 0.5 mgL\(^{-1}\). However, with 0.25 mgL\(^{-1}\) NAA alone, on an average 2.4 offshoots were formed. Contrary to embryonal axis explants, the shoot apices did neither callus at higher concentration of 1.0 mgL\(^{-1}\) BAP nor with the higher concentration of 0.25 mgL\(^{-1}\) NAA used in combination with 0.5 mgL\(^{-1}\) BAP.

In the present study, results obtained with shoot apices in respect of offshoot formation had been far better as compared to embryonal axis explants, which explant has mostly been investigated by a number of workers in grain legume tissue culture\(^{8,20-22}\). Shoot apices, therefore, may prove more useful in transgenic investigations with \(V.\, mungo\) in particular and grain legumes in general, since besides being regenerative, the offshoots formed are also expected to be pathogen-free if the explant size is small enough to comprise only the meristem dome and 1 to 2 young leaf primordia\(^{22}\). Further more, the constituent cells of shoot apices are genetically uniform, where introduction of foreign genes is more advisable in such genetically homogenous cell system since genetic modification will not be further modified due to genetic variability obtained in the explant itself.

Shoot tip explants as well as explants of embryonal axis when subcultured along with regenerated offshoots in a treatment containing 0.1 mgL\(^{-1}\) BAP, 0.25 mgL\(^{-1}\) IAA and 100 mgL\(^{-1}\) meso-inositol and in the other one comprising 0.1 mgL\(^{-1}\) BAP, 0.25 mgL\(^{-1}\) IAA and 200 mgL\(^{-1}\) casein hydrolysate (CH) showed better growth of shoots in the former treatment, which response was more in the case of shoot apices than the embryonal axes (Figs 1, 2). Shoots continued to grow individually while some shoot buds were also formed from their base, the growth of which was not inhibited by earlier formed shoots. In most of the cultures, the differentiation of shoot buds and their growth into shoots was continuous and a nonsynchronous process. However, after an incubation of 15 to 20 days, the shoot growth was restricted, while some callus tissue also started forming at the base of the groups of shoots. The good growth of shoots could only be restored if such cultures were transfered to the same treatment supplemented with 1.0 mgL\(^{-1}\) CCC. A
slightly less growth associated with callusing was obtained in higher concentrations of CCC, i.e., 2.5 or 5 mgL⁻¹, while growth was inhibited at a still higher concentration of 7.5 mgL⁻¹. However, best results were observed in a sequential culture of groups of regenerated shoots, initially for 7 days in 5 mgL⁻¹ CCC followed by their transfer to its 1 mgL⁻¹ concentration, while rest of the constituents of the medium remained the same. The growth promoting response of CCC is contrary to its classification amongst growth inhibitors, but it has been found to promote differentiation of offshoots in many of the plant systems. Morphogenetic competence of CCC has been mainly because of its antigibberellin action. It has been shown to augment regenerant promotion by a low concentration of ABA26. In the same medium, the cultures of proliferating shoots yielded 4 to 5 developed shoots after an incubation of 20 days. The developed shoots measuring about 4.0 cm in length and comprising at least 2 nodes were excised and rooted in the sole presence of 0.5 mgL⁻¹ IAA within a period of 10 days (Fig. 3). The rooted shoots were acclimatized after their direct transfer to potted Soil-rite and they were maintained in high humidity for the initial period of 5 days, which on transplantation to a potting mixture comprising 1:3 compost and garden soil grew normally and vigorously (Fig. 4). The in vitro-raised plants were kept under culture room conditions for about 15 days and finally transferred to grow under field conditions. It took about 30 days to raise a plant of an average height of 15 cm in potted soil from the time of culture of isolated shoots for rooting. The in vitro-raised plants came to flowering under field conditions after about 25 days of transplantation.

It has been shown in the present study that high frequency shoot multiplication leading to plantlet formation can be obtained from cultured shoot apices of Vigna mungo, on which very few reports are available. The in vitro-raised plants showed normal ex vitro growth under field conditions. The results can be of help for undertaking genetic transformation studies in this important grain legume, which has been found recalcitrant to regenerate in vitro, but to have high propensity of callusing instead.

Acknowledgement
The authors are thankful to the Director, N.B.R.I., Lucknow for providing the facilities.

References


