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Effect of Auxins and Cytokinins on Efficient Plant Regeneration and Multiple-Shoot Formation from Cotyledons and Cotyledonary-Node Explants of Groundnut (*Arachis hypogaea* L.) by In Vitro Culture Technology

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ABSTRACT

Tissue cultures were established from cotyledon and cotyledonary-node segments of *Arachis hypogaea* L. on Murashige and Skoog (MS) medium supplemented with different concentrations of auxins (IAA, NAA, IBA, and 2, 4-D) and cytokinins (KIN and BAP). For callus initiation, high concentration of auxins and low concentration of cytokinins were used, whereas high concentration of cytokinins and low concentration of auxins were used for shoot-bud differentiation. Callus induction and shoot-bud regeneration frequency, however, varied with genotype, explant, and the different plant-growth regulators combination in the medium. The shoot-bud multiplication was also influenced by genotype, explant type, and growth regulators. The combination of BAP and NAA produced more shoots than other combinations. The maximum number of shoots was obtained from cotyledonary-node segments on a medium containing BAP (5.0 mg/L) and IBA (1.0 mg/L). Rooting of regenerated shoots was achieved on a medium augmented with NAA or IBA (2.0 mg/L) in combination with KIN (0.5 mg/L). Rooted plantlets were successfully established in the soil, where 95% of them survived.

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Tissue-culture studies of these explants suggests the shoots to be of *de nova* origin, which would make the system suitable for gene-transfer technology.

Index Entries: *Arachis hypogaea;* in vitro technology; cytokinins; auxins; multiple shoot regeneration; organogenesis.

Abbreviations: MS, Murashige and Skoog (1962) medium; B5 vitamins, Gamborg et al. (1968); NAA, α-naphthaleneacetic acid; IAA, indole-3-acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; BAP, 6-benzylaminopurine; KIN, kinetin.

INTRODUCTION

Arachis hypogaea L., commonly known as groundnut or peanut, is an important oil seed and cash crop in warm tropical and subtropical regions throughout the world. The successful exploitation of in vitro techniques in plant biotechnology depends on the establishment of efficient plant-regeneration systems *(1)*. An efficient plant-regeneration system is also a prerequisite for genetic transformation studies using *Agrobacterium tumefaciens (2)*. The cultivated peanut, one of the grain-legume species, is recalcitrant in tissue culture *(3)*, although some successful plantlet regeneration has been achieved either by organogenesis or somatic embryogenesis *(3–14)*. However, the process of plant regeneration in these studies was slow and produced generally low frequencies of shoot buds. Data on the efficiency of regeneration in terms of the number of plantlets obtained, duration, and the response of different genotypes is lacking in the existing reports. In this investigation, we describe an efficient protocol for high frequency of plant regeneration and multiple shoot-bud production from cotyledon and cotyledonary-node explants after cutting of its surface layers to remove the pre-existing shoot meristems completely. A novel method of application of an auxin IBA for the continued shoot-bud initiation from the nodal explants has been described.

MATERIALS AND METHODS

Plant Material

Seeds of groundnut cultivars, VRI-2 and TMV-7 (obtained from the Tamil Nadu Agricultural University, Coimbatore, India), were used in the experiments. Aseptic seedlings served as the source of cotyledon and cotyledonary-node explants. Seeds were rinsed in Tween-80 for 5 min and surface sterilized in 0.1% (W/V) aqueous mercuric chloride for 7–10 min. After rinsing 4–5 times in sterile distilled water, 5–8 seeds were aseptically

implanted per 50 mL of MS *(15)* basal medium containing 2% (W/V) sucrose and 0.7% (w/v) agar in 250 mL Erlenmeyer flasks, plugged with nonabsorbant cotton, and wrapped in one layer of cheesecloth. The media was adjusted to pH 5.8 with 0.1 *N* NaOH or HCl, before autoclaving at 1.06 kg cm^2 pressure and 121°C temperature for 15 min. The seeds were allowed to germinate in 8 h dark and 16 h cool-white fluorescent light of 30–40 $\mu E\ m^{-2}s^{-1}$, at 25 ± 1.5°C for 7 d.

Preparation of Explants

The cotyledon explants without embryogenic axis (5–10 mm) were excised from 7-d-old seedlings and cultured on MS medium by embeeding 1 mm of the cotyledon in the medium. The cotyledonary-node explants, without two cotyledons excised from 7-d-old seedlings, were the most responsive for multiple-shoot formation. The effect of explants on shoot-bud regeneration response was studied by culturing on MS medium with various growth regulators.

Growth Regulators

The requirements of explants for exogenous hormones were examined. To assess the influence of different concentrations and combinations of IAA, NAA, IBA, 2,4-D, BAP, and KIN on production of callus, shoots, and roots, both explants (cotyledon and cotyledonary-node) were excised from 7-d-old seedlings and cultured on MS medium supplemented with B5 vitamins *(16)*, different concentrations, and combinations of IAA, NAA, IBA, 2,4-D, BAP, and KIN (0.5–2.0 mg/L). The multiple-shoot formation response of BAP (1.0–5.0 mg/L) was also compared with KIN.

Rooting and Transplantation

After a week, the regenerated shoots (3–5 cm long) were excised, counted, and cultured on MS medium augmented with NAA or IBA (0.5–2.0 mg/L) in combination with 0.5 mg/L KIN for rooting. Plantlets with well-developed roots were removed from the culture tubes and, after washing roots in running tap water, were transferred to plastic cups containing sand, red soil, and manure in the ratio 1:1:1. Polyethylene bags were over the plants to ensure high humidity during the first few days. After 15 d, plantlets were shifted to the field.

Culture Conditions

All cultures were maintained at 25 ± 2°C under 16/8 h light/dark conditions of 80 $\mu E\ m^{-2}s^{-1}$ irradiance provided by fluorescent lamps (TL 40 W/54 cool day light).

Observations of Culture and Presentation of Results

Usually, 20–24 cultures were used per treatment and each experiment was repeated at least three times. Visual observations of cultures were taken every week and the frequency of cultures showing shoot-bud differentiation and the number of shoots per culture were recorded 30 d after culture initiation. The experimental design was completely randomized design and factorial with auxin and cytokinin as independent variables. An analysis of variance (ANOVA) was conducted to evaluate the effect of auxin and cytokinin type on percent organogenic response. A Duncan's new multiple range test (DNMRT) was performed for each auxin and cytokinin to determine the effect of concentration on this response.

RESULTS AND DISCUSSION

Morphogenetic Response of Cotyledons and Cotyledonary-Node Explants on Callus Induction

Both cotyledon and cotyledonary-node explants were cultured on MS medium supplemented with different concentrations of IAA, NAA, IBA, and 2,4-D (0.5–2.0 mg/L) in combination with KIN (0.5 mg/L) for callus induction. The explants expanded to twice in their original size, and also formed greenish callus where the bud primordia did not. These calluses did not differentiate into shoot-bud primordia after a month of culture in the same medium, but 95.0% of the explants formed calluses in this medium in the first month of culture. The highest frequency of callus induction was 94.3 and 86.5% observed in cotyledon and cotyledonary-node, respectively, in VRI-2 cultivar, whereas it was 86.1 and 79.3% in cotyledon and cotyledonary-node, respectively, with TMV-7 cultivar on MS medium containing 2.0 mg/L NAA and 0.5 mg/L KIN. The IAA (0.5–2.0 mg/L) and KIN (0.5 mg/L) combination induced various frequencies of callusing i.e., 56.4–80.6% and 55.3–78.3% in cotyledon and cotyledonary-node, respectively, with VRI-2 cultivar, whereas it was 54.5–78.4% and 50.4–73.4% in cotyledon and cotyledonary-node, respectively, in TMV-7 cultivar. In VRI-2, callus induction was varied from 54.5–78.6% and 50.3–68.6% in cotyledon and cotyledonary-node, respectively, whereas in TMV-7, it was 52.3–73.5% and 51.3–67.9% in cotyledon and cotyledonary-node, respectively, with different concentrations of IBA (0.5–2.0 mg/L) in combination with 0.5 mg/L KIN. Callus initiation from cotyledon explant varied from 49.5–68.3% and 45.3–64.7% in VRI-2 and TMV-7 cultivar, respectively, whereas in cotyledonary-node, it was varied from 45.4–65.5% and 44.5–64.1% in VRI-2 and TMV-7 cultivar, respectively, with various concentrations of 2,4-D (0.5–2.0 mg/L) in combination with KIN (0.5 mg/L).

In the present study, we have obtained high frequency of plant regeneration from both cotyledon (without cotyledonary-node) and cotyledonary-node (without embryo axis) explants through callus-culture technology. Results obtained from this experiment revealed that groundnut cotyledon, as well as cotyledonary-node explants, were found to be equally suitable for callus formation. However, cotyledon explant produced highest frequency of callusing than cotyledonary-node explant. Of the four auxins tested for their callusing ability, NAA was observed, in general to be more effective than other three auxins for callus initiation. The effect of NAA on callus initiation was significantly different from other auxins at 1% level. Among the different concentrations of NAA tried, 2.0 mg/L was found to be the best concentration for maximum frequency of callus induction. Both auxin type and concentration have a significant effect on the frequency of callus induction per responding explant (1% level). A comparison of the relative effectiveness of different auxins for callus formation revealed in order of effectiveness NAA > IAA > IBA > 2,4-D. Data on callus induction from both the explants with two cultivars on MS media showed some variation. The results showed that explants, genotypes, and hormone combinations and concentrations play an important role in callus development. Moreover, the combination of auxin in higher concentration and cytokinin in lower concentration is more effective for callus induction. This observation is in agreement with previous work on *Arachis hypogaea (11,14), Vigna radiata (17,18), Guizotia abyssinica (19), Cajanus cajan (20).*

Shoot-Bud Regeneration

After 3 wk, these calluses were transferred to MS medium containing different concentrations of BAP (0.5–2.0 mg/L) in combination with various auxins namely IAA, NAA, IBA, and 2,4-D (0.5 mg/L) for shoot-bud regeneration. Calluses produced shoot-bud primordia and these buds developed into normal shoots after 1 mo of culture. The maximum frequency of shoot-bud differentiation was 89.9 and 100% in cotyledon and cotyledonary node, respectively, observed on MS medium containing BAP (2.0 mg/L) + NAA (0.5 mg/L) combination in VRI-2 cultivar, whereas it was 86.3 and 95.3% in cotyledon and cotyledonary node, respectively, in TMV-7 cultivar. Shoot-bud regeneration from cotyledon derived callus varied from 52.4 to 82.1% and 50.3 to 79.4% in VRI-2 and TMV-7, respectively, whereas in cotyledonary-node derived callus, it was 54.4 to 88.4% and 53.3–84.3% in VRI-2 and TMV-7, respectively, on a medium containing different concentrations of BAP (0.5–2.0 mg/L) in combination with 0.5 mg/L IAA. In VRI-2, shoot-bud differentiation was varied from 50.3–81.3% and 56.3–86.3% in cotyledon and cotyledonary-node, respectively, whereas in TMV-7, it was

varied from 46.3–77.2% and 49.3–81.3% with cotyledon and cotyledonary-node, respectively, on a medium augmented with various concentrations of BAP (0.5–2.0 mg/L) in combination with 0.5 mg/L IBA. The BAP (0.5–2.0 mg/L) and 2,4-D (0.5 mg/L) combination produced various frequencies of shoot-bud regeneration, i.e., 50–77.3% and 45.3–74.4% in VRI-2 and TMV-7, respectively, with cotyledon-derived callus and in cotyledonary node-derived callus, it was varied from 51.6–81.3% and 47.3–79.4% in VRI-2 and TMV-7, respectively.

These shoot-bud initials were subcultured to the shoot-bud multiplication medium containing either BAP or KIN (1.0–5.0 mg/L) in combination with IBA (1.0 mg/L). In cotyledon, the number of multiple shoots varied from 6.4 to 32.5 and 5.3 to 25.4 shoots/culture in BAP + IBA and KIN + IBA combination, respectively, whereas in cotyledonary-node, it was 10.5 to 35.4 and 8.4 to 30.6 shoots/culture in BAP + IBA and KIN + IBA combination, respectively, in VRI-2 cultivar. In TMV-7 cultivar, the number of multiple shoots varied from 5.9 to 30.9 and 9.8 to 31.3 shoots/culture in cotyledon and cotyledonary-node, respectively, on a medium containing different concentrations of BAP (1.0–5.0 mg/L) in combination with 1.0 mg/L IBA whereas it was varied from 4.5 to 22.1 and 6.8 to 25.6 shoots/ culture in cotyledon and cotyledonary-node, respectively, with KIN and IBA combination.

The cytokinin BAP, has been commonly used for shoot-bud induction in legumes. The involvement of cytokinins especially in shoot-bud formation has been reported in many plant species. On the other hand, cotyledonary segments produced multiple shoots in the presence of either KIN or BAP *(19)*. The combination of auxins and cytokinins at definite proportions are very critical and found to be essential for the induction of shoot and root in many species *(21–25)*. The cytokinins (BAP and KIN) enhanced multiple shoot-bud regeneration in cultured cotyledon and cotyledonary-node explants of *Arachis hypogaea* is in accordance with previous reports on grain legumes *(17,18,20)*. The maximum response (100%) of cultures for shoot-bud differentiation was in MS medium supplemented with NAA (0.5 mg/L) + BAP (2.0 mg/L) which was significantly different at the 1% level. Of the two cytokinins tested, BAP (5.0 mg/L) was produced maximum number of shoots (35.4) per culture which was highly significant (1% level). In the absence of BAP from the medium, no induction of shoot-bud regeneration was observed. The important role of BAP for shoot-bud differentiation in legumes has been reported previously *(14,17,18,20)*. Our results indicated that the addition of NAA increased the frequency of shoot-bud regeneration and root formation, are consistent with results of *Brassica napus (25,26)*. The classical findings in tissue cultures is governed by the balance of auxin and cytokinin present in the medium which cannot be demonstrated universally owing to the explant sensitivity or the original content of endogenous-growth regula-

tors. It is, therefore, organ differentiation occurs from explants depends upon the endogenous-growth regulators. This suggests that the minimum amount of hormones should be supplemented exogenously for shoot bud differentiation.

Proliferation of multiple shoot-initials from the cultures only in the presence of added IBA suggests that the cytokinin:auxin ratio is important for this response. Topical IBA application was observed to play a critical role in the formation of new-shoot initials from the shoot cultures. Though the underlying mechanism for this response needs to be investigated, it could be attributed to the availability of small quantity of IBA being mainly restricted to the regenerating surface of the explant.

Root Initiation

After a mo of culture, the elongated shoots that attained 3–5 cm long were excised and transferred to MS medium supplemented with different concentrations of NAA or IBA (0.5–2.0 mg/L) in combination with KIN (0.5 mg/L) for rooting. Roots emerged from the cut end of the shoots within two wk of culture. The root-induction frequency was high (83.4 and 78.8% in cotyledon and cotyledonary-node, respectively) in VRI-2 cultivar, whereas it was 76.3 and 77.6% in cotyledon and cotyledonary-node respectively, with TMV-7 cultivar on a medium fortified with IBA (2.0 mg/L) in combination with 0.5 mg/L KIN. In VRI-2 cultivar, the percentage of rooting was varied from 47.4–76.5% and 46.3–75.3% in cotyledon and cotyledonary-node, respectively, whereas in TMV-7 cultivar, it was varied from 38.8–71.2% and 41.6–74.9% in cotyledon and cotyledonary-node, respectively, on a medium containing various concentrations of NAA (0.5–2.0 mg/l) in combination with 0.5 mg/L KIN. The rooted shoots from both the explants (cotyledon [Fig. 1] and cotyledonary-node [Fig. 2]) were transferred to plastic cups and later established in glasshouse, where 95% of them survived and resumed growth. Regenerated plantlets from both the explants produced normal pods with viable seeds.

The differentiated shoots required NAA or IBA along with KIN for root initiation. In the present study, IBA was found to be more effective for high frequency of root initiation. The highest frequency of rooting was recorded on a medium containing 2.0 mg/L IBA in combination with 0.5 mg/L KIN, where all shoots formed roots within two wk of culture and this concentration was significantly different from other concentrations at 1% level. Two mg/L NAA was also effective for root induction (75% of shoots rooted), the root system, however, was quantitatively poorer than that grown in 2.0 mg/L IBA medium. Similar observations were made by Moss et al. *(27)*, Narasimhulu and Reddy *(11)*, Cheng et al. *(3)*, Venkatachalam et al. *(5)*, Ono et al. *(25)*.

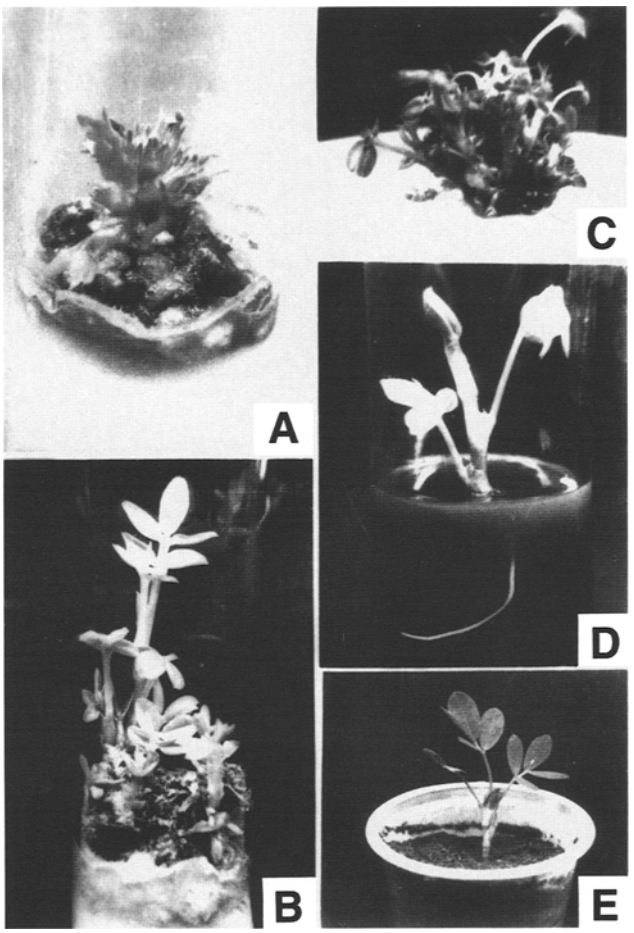

Fig. 1. Plant regeneration from cotyledon explant. **(A)** Differentiation of shoot buds from cultured cotyledons of groundnut cultured on MS + BAP (2.0 mg/L) + NAA (0.5 mg/L). **(B)** Shoots developed from the cotyledon explant-derived callus. **(C)** Cotyledon derived callus showing multiple shoot-bud regeneration on MS + BAP (5.0 mg/L) + IBA (1.0 mg/L). **(D)** A groundnut 4-wk-old shoot showing root formation on a medium with 2.0 mg/L IBA + 0.5 mg/L KIN + 3% activated charcoal. **(E)** A regenerated plant established in plastic cup containing soil.

Effect of Different Auxins

The effect of different concentrations (0.5–2.0 mg/L) of auxins viz., NAA, IAA, IBA, and 2,4-D was studied on callus induction, shoot-bud regeneration, shoot-bud multiplication, and root formation. Among these auxins tested, NAA was found to be superior for callus induction and shoot-bud regeneration, whereas IBA was found to be potent auxin for shoot-bud multiplication and root initiation followed by IAA and 2,4-D. The shoots formed

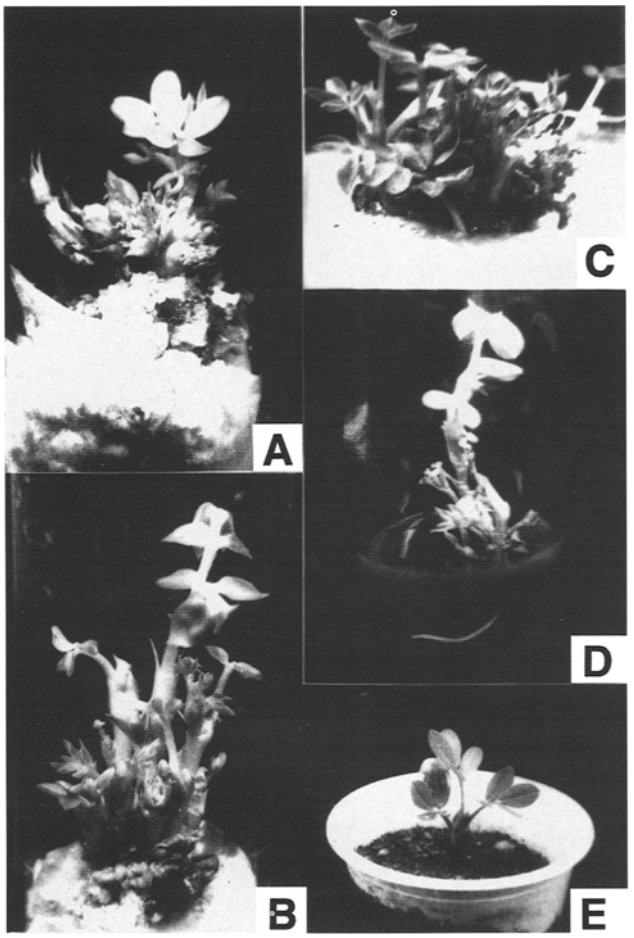

Fig. 2. Plant regeneration from cotyledonary node explant. **(A)** Cotyledonary-node explant showing shoot organogenesis on MS + BAP (2.0 mg/L) + NAA (0.5 mg/L). **(B)** Shoot buds developed from the cotyledonary-node derived callus. **(C)** Multiple-shoot production from cotyledonary-node derived callus cultured on MS + BAP (5.0 mg/L) + IBA (1.0 mg/L). **(D)** Regenerated shoot rooted on MS + IBA (2.0 mg/L) + KIN (0.5 mg/L) + 3% activated charcoal. **(E)** Well-developed groundnut plantlet acclimatized in plastic cup containing soil.

on these auxin containing media were more robust than those on media without auxin or with BAP or KIN. The efficacy of BAP or KIN for shoot-bud multiplication was increased when it was supplemented with IBA.

Effect of Different Cytokinins

Because the maximum number of shoots per culture was observed with 5.0 mg/L BAP, hence this concentration was chosen to study the comparative response of two cytokinins. Both BAP and KIN favored

multiple shoot-bud development from shoot cultures. BAP not only induced differentiation of shoots but in 95% of the total cultures, shoots also subsequently developed into multiple shoots. However, the number of shoots per culture varied with cytokinin. BAP was the most effective and best cytokinin than KIN with regard to the number of shoots per culture.

Effect of BAP Concentration

Addition of BAP also enhanced the frequency of shoot bud regeneration as well as the number of shoots per culture. Addition of different concentrations of BAP to MS medium induced high frequency of shoot-buds, followed by multiple-shoot formation from the callus cultures. If increase the concentrations of BAP, the number of shoots also increased considerably. BAP at 5.0 mg/L induced the maximum number of shoots (35.4 shoots/culture) in 100% of cultures.

Effect of Genotype

Using optimized conditions for callus induction, shoot-bud regeneration, multiple-shoot production, and root formation as previously described, two cultivars of groundnut were examined regarding organogenesis response. Of the two cultivars tested, VRI-2 was found to be more responsive cultivar for organogenesis. However, there was some quantitative variation in regeneration responses between both the cultivars.

The regeneration procedure was tested to determine its applicability to other groundnut or peanut cultivars. Genotype was not found to exert a pronounced effect on regeneration frequency. However, VRI-2 cultivar was found to be superior for tissue-culture studies than TMV-7 cultivar. This might be owing to the hybrid cultivar. Cheng et al. *(3)* reported that the hybrid cultivar was found to be best for in vitro studies. Similar results were also observed in the present study.

We conclude that *A. hypogaea* cotyledons and cotyledonary-nodes carry a high potential for rapid multiple shoot-bud regeneration via callus culture. This system may be useful for clonal multiplication of individual genotypes; and may have potential use for DNA transfer technology in *A. hypogaea*, which so far has been limited *(28)*. In addition, this cotyledon and cotyledonary-node system has been efficiently used recently for *Agrobacterium*-mediated gene-transfer technology in other legumes with high-transformation frequencies.

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REFERENCES

1. Hazra, R. B., Sathaye, S. S., and Mascarenhas, A. F. (1989), *Biotechnology* **7,** 949–951.
2. Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. (1985), *Science* **227,** 1229–1231.
3. Cheng, M., Hsi, D. C. H., and Phillips, G. C. (1992), *Peanut Sci.* **19,** 82–87.
4. Kartha, K. K., Pahl, K., Leung, N. L., and Mroginski, L. A. (1981), *Can. J. Bot.* **59,** 1671–1679.
5. Venkatachalam, P., Pillai, A. S., and Jayabalan, N. (1994), *Proc. Nat. Acad. Sci.* **64,** 99–103.
6. Mroginski, L. A., Kartha, K. K., and Shyluk, J. P. (1981), *Can. J. Bot.* **59,** 826–830.
7. Pittman, R. N., Banks, D. J., Kirby, J. S., Mitchell, E. D., and Richardon, P. E. (1983), *Peanut Sci.* **10,** 21–25.
8. Dunbar, K. B. and Pittman, R. N. (1992), *Crop Sci.* **32,** 1353–1356.
9. Eapen, S. and George, L. (1993), *Plant Cell Tiss Org. Cul.* **35,** 223–227.
10. Bajaj, Y. P. S., Kumar, P., Labana, K. S., and Singh, M. M. (1981), *Indian J. Exp. Biol.* **19,** 1026–1029.
11. Narasimhulu, S. B. and Reddy, G. M. (1983), *Plant Sci. Lett.* **31,** 147–153.
12. Atreya, C. P., Papa Rao, J., and Subramanyam, N. C. (1984), *Plant Sci. Lett.* **34,** 379–383.
13. Mc Kently, A. H., Moore, G. A., and Gardner, F. P. (1990), *Crop Sci.* **30,** 192–196.
14. Daimon, H. and Mii, M. (1991), *Japan J. Breed.* **41,** 361–366.
15. Murashige, T. and Skoog, F. (1962), *Physiol. Plant.* **15,** 473–497.
16. Gamborg, O. L., Miller, R. A., and Ojima, K. (1968), *Exp. Cell Res.* **50,** 151–158.
17. Gulati, A. and Jaiwal, P. K. (1994), *Plant Cell Rep.* **13,** 523–527.
18. Gulati, A. and Jaiwal, P. K. (1990), *Plant Cell Tiss. Org. Cul.* **23,** 1–7.
19. Ganapathi, T. R. and Nataraja, K. (1993), *Biol. Plant.* **35,** 209–215.
20. Shiva Prakash, N., Pental, D., and Bhalla-Savin, N. (1994), *Plant Cell Rep.* **13,** 623–627.
21. Lazzeri, P. A. and Dunwell, J. M. (1986), *Ann. Bot.* **58,** 689–697.
22. George, L., Bapat, V. A., and Rao, P. S. (1987), *Ann. Bot.* **60,** 17–21.
23. Reddy, K. R. K. and Bahadur, B. (1989), *Curr. Sci.* **58,** 152–154.
24. Shankar, S. and Mohan Ram, H. Y. (1990), *Curr. Sci.* **59,** 39–43.
25. Ono, Y., Takahata, Y., and Kaizuma, N. (1994), *Plant Cell Rep.* **14,** 13–17.
26. Sharma, K. K., Bhojwani, S. S., and Thorpe, T. A. (1990), *Plant Sci.* **66,** 247–253.
27. Moss, J. P., Dutt, N. R. G., and Lingamaneeni, A. (1988), *Inter. Arachis News Lett.* **4,** 25–26.
28. Eapen, S. and George, L. (1994), *Plant Cell Rep.* **13,** 582–586.