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Multiple shoot regeneration in seed-derived immature leaflet explants of peanut (*Arachis hypogaea* L.)

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ABSTRACT

A protocol was developed for organogenesis from immature leaflet explants derived from mature seeds of peanut. Immature leaflets pre-incubated on MS medium supplemented with 13.32 μM BAP + 4.95 μM NAA for 7 days, turned green and enlarged. The enlarged green leaflets produced multiple shoot buds after 1–2 cycles of sub-culture on MS medium supplemented with 13.32 μM BAP. Three cycles of shoot buds on the elongation medium (13.32 μM BAP) produced 6.17 ± 0.47 elongated shoots per explant. The shoot bud formation was genotype independent. All elongated shoots rooted on the medium containing 4.95 μM NAA. The complete protocol gave efficient (>81%) direct organogenesis, leading to the development of plantlets within 4 months.

1. Introduction

Peanut or groundnut (*Arachis hypogaea* L.) has captured the attention of several researchers due to its highly nutritive value as a source of protein and oil. The crop suffers from many biotic and abiotic stresses which cause major losses in terms of quality and quantity. Conventional breeding has contributed towards the improvement of peanut (Reddy et al., 1996; Garcia et al., 2006) but strong interspecific barriers, low recovery of hybrids and linkage of undesirable traits limit the introgression of variability from wild species (Halward et al., 1993; Tallury et al., 2005). Genetic transformation overcomes these limitations and allows introduction of agronomically important genes across taxa (Li et al., 1997; Singsit et al., 1997; Yang et al., 1998; Magbanua et al., 2000; Sharma and Anjaiah, 2000; Livingstone et al., 2005; Tiwari et al., 2008). The availability of an efficient regeneration system is however an essential prerequisite for utilizing this approach.

Immature leaflets from young seedlings of peanut had been utilized to achieve organogenesis (Mroginski et al., 1981; McKently et al., 1991; Cheng et al., 1992; Sukumar and Sree Rangasamy, 1984; Narasimhulu and Reddy, 1983; Akasaka et al., 2000; Chengalrayan et al., 2001). However, among those reports some had shown low regeneration efficiency and excessive time required for the development of the regenerants. For instance, Mroginski et al. (1981) and Cheng et al. (1992) reported bud primordia development that failed to regenerate normal plants. Sukumar and Sree Rangasamy (1984) reported calli development in seven *Arachis* spp. but none of them formed shoots. Narasimhulu and Reddy (1983) reported callus mediated plant development in only 19 % of the explants. Akasaka et al. (2000) reported various abnormalities in shoot development and low conversion rate (34.7%) from shoot buds to shoots. Mature zygotic embryo derived leaflet explants have been deployed by Chengalrayan et al. (2001) to examine the effect of phytohormones on organogenesis and somatic embryogenesis.

The present paper reports *in vitro* direct organogenesis from immature leaflet explants derived from zygotic embryos of mature seeds. Mature dry seeds guarantee year-round availability of explants for continuous research. The proposed protocol could improve peanut micropropagation with the potential of enhancing exogenous gene transfer.

2. Materials and methods

2.1. Plant materials and explant preparation

The pods of four peanut cultivars extensively cultivated in southern states of India viz., JL-24 (Maharastra and Karnataka), TMV-2 (Tamil Nadu and Karnataka), TAG-24 (Maharastra, Karnataka and Tamil Nadu) and Dh-3-30 (Karnataka) were obtained from the University of Agricultural Sciences, Dharwad (Karnataka, India). Mature dry seeds were surface-sterilized (Tiwari and Tuli, 2008) in 0.1% aqueous mercuric chloride for 10 min, rinsed 6–7 times with sterile water and left soaked overnight in sterile water.

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Abbreviations: BAP, 6-benzylaminopurine; MS, Murashige and Skoog (1962) medium; NAA, α-naphthalene acetic acid.

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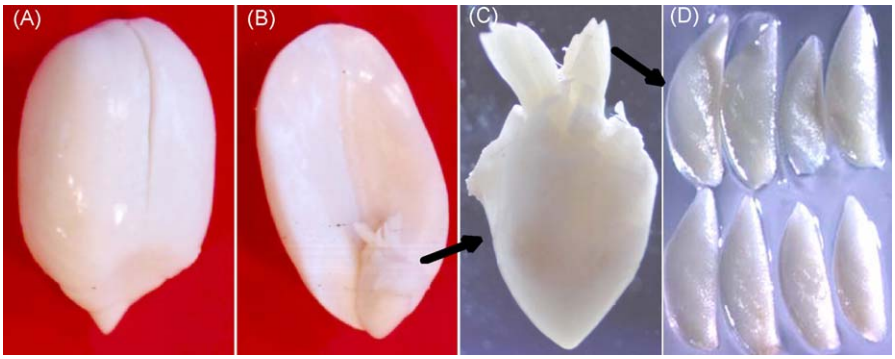

Fig. 1. Stages in the preparation of immature leaflet explants. (A) Seed coat peeled off from pre-soaked sterilized seed. (B) Embryonated cotyledon. (C) Embryo axis excised by removing the cotyledon (arrow). (D) Immature leaflet lamina used as explant (arrow).

The embryo axes were excised by removing the cotyledons aseptically from the sterilized seeds. The radical portion of embryo axis was cut out and the immature leaflet lamina was used as explant for formation of multiple shoots (Fig. 1).

2.2. Regeneration via organogenesis

Immature leaflets were cultured in petridish (94 mm diameter), on semi-solid medium containing MS salts (Murashige and Skoog, 1962), B_5 vitamins (Gamborg et al., 1968), 100 mg/l myo-inositol and 30 g/l sucrose supplemented with 6-benzylaminopurine (BAP) (13.32 μM) and α-naphthalene acetic acid (NAA) (4.95, 9.90 and 14.85 μM). Twenty to twenty five leaflets were cultured for pre-incubation in each petridish. Three petridishes, containing a total of 60–75 explants were cultured and incubated for 7, 15 and 30 days. There were three replications in each experiment. Percentage of explants showing shoot development response was noted. After pre-incubation, the explants in the three lots were transferred to shoot formation and elongation medium. The shoot formation and elongation medium contained MS medium supplemented with 13.32 μM BAP. Shoot buds appeared on the explants within three weeks and increased in number to make clusters. Percent response, mean and standard deviation in each experiment were calculated from data generated at this stage. Subsequently, shoot clusters were sub-cultured on similar medium every three weeks for enhancing shoot elongation. After 3–4 cycles, the number of elongated shoots was recorded. Shoots (3–4 cm) derived from the shoot clusters were excised and rooted on MS medium supplemented with NAA (4.95 μM).

2.3. Genotypic response

Immature leaflet explants prepared from four peanut cultivars were cultured for 7 days on pre-incubation medium and transferred on shoot formation and elongation medium (13.32 μM BAP) for 3 cycles of 3 weeks each. The multiple shoot bud formation (percent ± SD) and shoot elongation (mean number of elongated shoots per explant ± SD) efficiency were compared.

2.4. Culture conditions and hardening

Throughout the study, the cultures were incubated at 25 ± 2 °C in 80 μmol photon m^{-2} s^{-1} light intensity with a photoperiod of 16/8 h. All the media were solidified with 0.8% agar. The pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min. The hardening of the regenerated plantlets was carried out with irrigation in plastic pots containing Soilrite mix (Keltech Energies Ltd., Bangalore, India). Pots were initially kept under plexiglass acclimatization hoods (Basco Pvt. Ltd., India) with 85% relative humidity for 15 days. For the next 15 days, the hoods were raised gradually, decreasing the humidity. After 4 weeks, the plants were planted in sandy loam soil and kept in a glass-house till maturity.

All biochemicals and media constituents, unless stated otherwise were molecular biology/cell culture grade from Sigma Chemical Company (St. Louis, MO, USA).

3. Results and discussion

The present report describes a rapid, reproducible and efficient protocol for *in vitro* propagation of peanut. Earlier reports on *in vitro* organogenesis in peanut showed strong influence of genotype (Mroginski et al., 1981; Seitz et al., 1987; McKently et al., 1990; Cheng et al., 1992; Banerjee et al., 2007; Matand and Prakash, 2007) and culture conditions (Chengalrayan et al., 1995; Akasaka et al., 2000; Palanivel and Jayabalan, 2002; Vasanth et al., 2006; Tiwari and Tuli, 2008) on the response of explants.

Immature leaflet explants of peanut were reported to respond significantly in organogenesis (Mroginski et al., 1981; Cheng et al., 1992; Sukumar and Sree Rangasamy, 1984; Narasimhulu and Reddy, 1983; Seitz et al., 1987; Akasaka et al., 2000; Chengalrayan et al., 1995, 2001). Mroginski et al. (1981) used leaflets at different developmental stages and exposed them to MS medium supplemented with 12 combinations of NAA and BAP. The results showed that immature leaflet explants were most responsive and gave highest bud formation in 1 mg/l each of NAA and BAP containing medium. Seitz et al. (1987) reported that immature leaves induced up to 30% shoot formation on the MS medium containing NAA (1 mg/l) and BAP (1 mg/l). Cheng et al. (1992) and Akasaka et al. (2000) reported that MS medium containing NAA and BAP was the most promising combination for shoot bud formation from immature leaflet explants. In the present study, the zygotic embryo derived immature leaflet explants pre-incubated for 15 and 30 days on MS medium supplemented with combinations of BAP (13.32 μM) and NAA (4.95 and 9.90 μM) showed very low frequency of shoot bud formation. The 13.32 μM BAP and 14.85 μM NAA combination gave no shoot buds (Table 1). Seven-day pre-incubated leaflet explants formed no shoot buds in any combination, though they turned green and enlarged in size (Fig. 2A). When the cultures pre-incubated for different days were transferred to shoot formation and elongation medium containing BAP (13.32 μM), the frequency of shoot bud formation was substantially increased (Table 2). Seven day pre-incubated leaflets showed highest percent of multiple shoot bud proliferation on the shoot formation and elongation medium. Shoot bud clusters sub-cultured on the similar medium resulted in enhanced shoot elongation. Therefore, the highest shoot bud formation (81.5 %) and shoot elongation (6.17 shoots/explant) were obtained when the explants pre-incubated for 7 days on medium containing 13.32 μM BAP and 4.95 μM