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Direct Regeneration from Leaf Disc Explants of Peanut: Grafting Improves Survival Rate

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Abstract:
Leaf discs of 0.5 cm diameter from 10 days old seedlings of four peanut cultivars were cultured on Murashige and Skoog (MS) medium supplemented with twelve different combinations of Thidiazuron (TDZ) and Naphthalene acetic acid (NAA). The highest number of responding explants (52.67%) with the highest number of shoot buds (5.65) per responding explant was achieved at a combination of 0.5 mg/l NAA and 0.5 mg/l TDZ. The explants were then shifted to fresh MS medium containing 8 mg/l Benzylaminopurine (BAP) and 0.5 mg Indole Acetic Acid (IAA) for shoot elongation. Root induction was highest (56.07%) at half-strength MS medium supplemented with 1.5 mg/l NAA. Grafting of in vitro regenerated shoots on 10 days old seedling rootstock showed highest (61.30%) success rate. The survival rate of plants obtained from grafting was almost double (45.76%) than those achieved by rooting of shoots (22.41%). There was significant variation among four varieties regarding the number of responding explant, the number of shoots per explant, rooting or grafting percentage and survival rate. Golden proved to be the best variety in terms of the number of plants reaching maturity.

Keywords: Grafting, *In vitro* regeneration, Leaf disc, Naphthalene acetic acid, peanut.

INTRODUCTION

In vitro regeneration in plants is accomplished either through embryogenesis or organogenesis. Complete procedure of organogenesis is less understood. The effects of genotype explant and plant growth promoters on somatic embryogenesis and organogenesis have been evaluated in many plant species. In model plants like tobacco, leaf discs are the most commonly used explants for *Agrobacterium* mediated and biolistic transformation (Horsch *et al.,* 1984). Mroginski et al. (1981) produced six varieties of groundnut plants through organogenesis from immature leaf culture. Many researchers have expanded this technique to include various genotypes of peanut (Cheng *et al.,* 1992; McKently, 1991; Seitz *et al.,* 1987; Utomo *et al.,* 1996). Similar work has been reported in other species of *Arachis* genus (McKently *et al.,* 1991; Pittman *et al.,* 1983). Regeneration through organogenesis has also been achieved using mature leaf discs in different species of *Arachis* (Burtnik and Mroginski, 1985; Rey *et al.,* 2000).

In vitro tissue culture response is governed by combination of internal hormones naturally present inside the explant tissue and those present in the medium (Evans *et al.,* 1981). Different species of *Arachis* have been regenerated by various combinations of 2, 4-Dichlorophenoxy acetic acid (2, 4-D) or Naphthalene acetic acid (NAA) and Kinetin (KIN) or Benzylaminopurine (BAP) (Mroginski and Kartha, 1984). However, some researchers have used thidiazuron in regeneration experiments of cultivated peanut (Akasaka *et al.,* 2000; Gill and Ozias-Akins, 1999). Thidiazuron has potent activity as a cytokinin in the induction of shoot organogenesis in several plant species (Murthy *et al.,* 1998; Mithila *et al.,* 2001).

The frequencies of regeneration were low and procedures involved multiple, laborious and time taking steps in most of previous experiments (Pacheco *et al.,* 2009; Joshi *et al.,* 2008). Present study was designed to optimize a reproducible and high-efficiency regeneration system for peanuts using young leaves in medium supplemented with Naphthaleneacetic acid and thidiazuron.

MATERIALS AND METHODS

Explant Preparation

Seeds of four groundnut cultivars viz. BARD-92, BARI-2000, BARD-479 and Golden were provided by Barani Agriculture Research Institute, Chakwal, Pakistan. Freshly de-shelled seeds were sterilized by treating with 70% (v/v) ethanol for one minute and then with 20% clorox (commercial bleach with 5.25% Sodium hypochlorite) for ten minutes. After rinsing with sterile water thrice, five seeds of each variety were inoculated in simple solidified autoclaved Murashige and Skoog (MS) medium (Murashige and Skoog 1962) for germination. Leaf discs of 0.5 cm diameter containing mid rib were cut from leaves of 10 days old seedlings under sterile conditions.

Medium and Culture Conditions

The leaf disk explants were cultured on MS salts, B5 vitamins (Gamborg *et al.,* 1968), 30 g/L sucrose supplemented with 12 different hormone combinations. Agar was added at the rate of 8 g/L after adjusting the pH at 5.8 at 25°C. The leaf disc explants were placed with their abaxial side in contact with medium at 25± 2°C with 16 hours light duration. Completely randomized design was used for this experiment which consisted of three batches each having 40 explants. The data on number of responding explants and shoots/explant were recorded after four weeks of culture.

After four weeks the responding discs were shifted to fresh MS medium containing 8 mg/l BAP and 0.5 mg IAA. On attaining a height of 5.0 cm, these shoots were cut and half of them were shifted to rooting medium and other half were used as scions in grafting.

The rooting medium consisted of half MS salts, B5 vitamins, 15g/L sucrose and three different concentrations of NAA viz. 0.5, 1.0 and 1.2mg/L. The rooted plants were transplanted in pots containing coconut husk compost. Pots were covered with polythene sheet to maximize the humidity for first seven days.

Half of the shoots were used for grafting on healthy 10, 15 and 20 days old root stocks of another commercially grown variety, Chikory. A wedge shaped 3cm long scion was inserted in a T-shaped cut made in seedling grown in 25 x 25 cm earthen pot. The joint was wrapped with parafilm and pot was covered with polythene bag which was removed gradually in 7 days.

Data Analysis

Analysis of variance (ANOVA) was performed using M-STATC software. Means of parameters showing significant variations were ranked by Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

To see whether treatments and varieties make some significant difference or not, analysis of variance (ANOVA) was performed. If treatments and varieties made significant differences, while their interactions showed no significance, DMRT was used for ranking of treatments and varieties only. If, however, interaction was also significant along with varieties or treatments, DMRT was applied on interaction only.

Number of Responding Explants (%age)

Number of explants showing shoot development has been referred as responding explants. After ten days of culture, it was observed that leaf disc was enlarged and thickened and callus was observed from cut surface (Figure 1). Shoot induction was observed after four weeks of the inoculation from cut ends of midrib. These findings were in agreement with Eapen and George, (1993) and Geng et al. (2011). Analysis of variance revealed that treatments, varieties and their interactions had highly significant impact on number of explants responding for shoot induction. Duncan's Multiple Range Test for interaction means showed that highest mean number of responding explants (52.67%) were obtained at Treatment No. 6 in Golden variety while lowest (1.00%) number of explants responded at treatment No. 1 and 9 in BARD-92 (Figure 2).

Akasaka et al. (2000) reported 34.7% response rate while Geng et al. (2011) observed that 40.9% explants responded to shoot induction on a medium containing thidiazuron. Difference in hormone combinations and genotypes might have contributed to this variation.

Number of Shoots/Responding Explants

Analysis of variance revealed that treatments, varieties and their interactions have highly significant impact on number of shoots/responding explants. Duncan's Multiple Range Test showed that highest (5.69) mean number of shoots/responding explants were obtained from the explants previously cultured on Treatment No. 6 in Golden variety while lowest value (1.00) was observed in those of Treatment No. 1 and 9 in BARI-2000 and BARD-479 respectively (Figure 3). Eapen and George (1993) observed 7.0 shoots/responding explant on average while Geng et al. (2011) reported 5.0 shoots /responding explant.

Rooting Percentage

The prime objective of the study was to get maximum number of rooted plants/explant as this will be an ultimate goal in any tissue culture based transformation procedure. Analysis of variance showed that treatments and varieties had highly significant impact on this parameter. 56.07% shoots developed roots at 1.5 mg/liter NAA while only 12.69% shoots developed roots at 0.5 mg/liter NAA. However root development achieved (55.82%) at 1.0

mg/liter NAA was non-significantly less than top value. Golden variety responded best to root induction (44.06%) while lowest (36.03%) root induction was observed in BARD-92. Verma et al. (2009) used 0.5, 1.0, 1.5, and 2.0 mg/l NAA for root inductions but observed root induction only at 1.0 mg/l NAA in four commercial Indian Peanut varieties. In our study 1.0 and 1.5 mg/l NAA gave excellent results, however, root induction was observed at all hormone levels used.

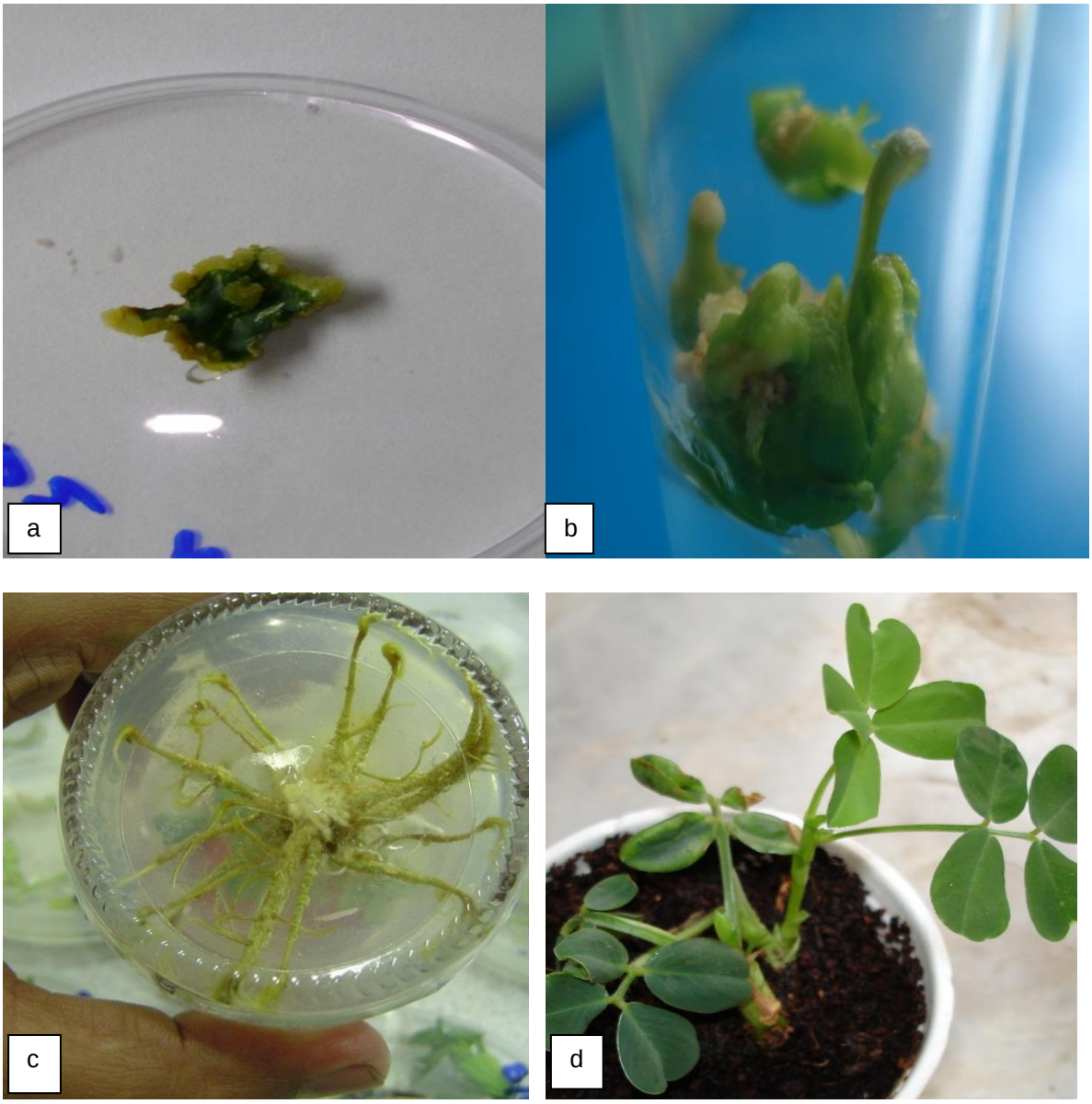

Fig. 1. Different steps of shoot induction from leaf discs**; a)** Increase in size, thickening and callus induction from cut surface of leaf disc after 10 days of culture; **b)** Development of shoot buds**; c)** Development of roots**; d)** Plantlet growing in coconut husk peat

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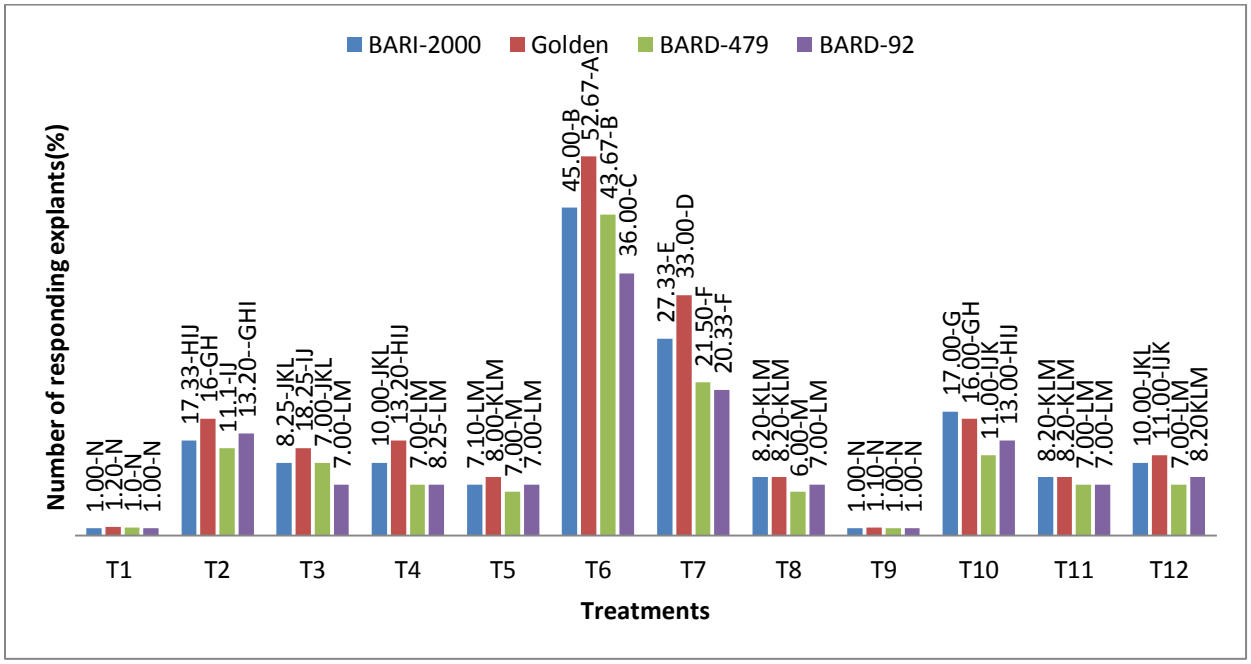

Fig. 2. Duncan's Multiple Range Test for ranking of interaction means with respect to number of responding explants (%). Values sharing same letters do not differ significantly at 5% level of probability.

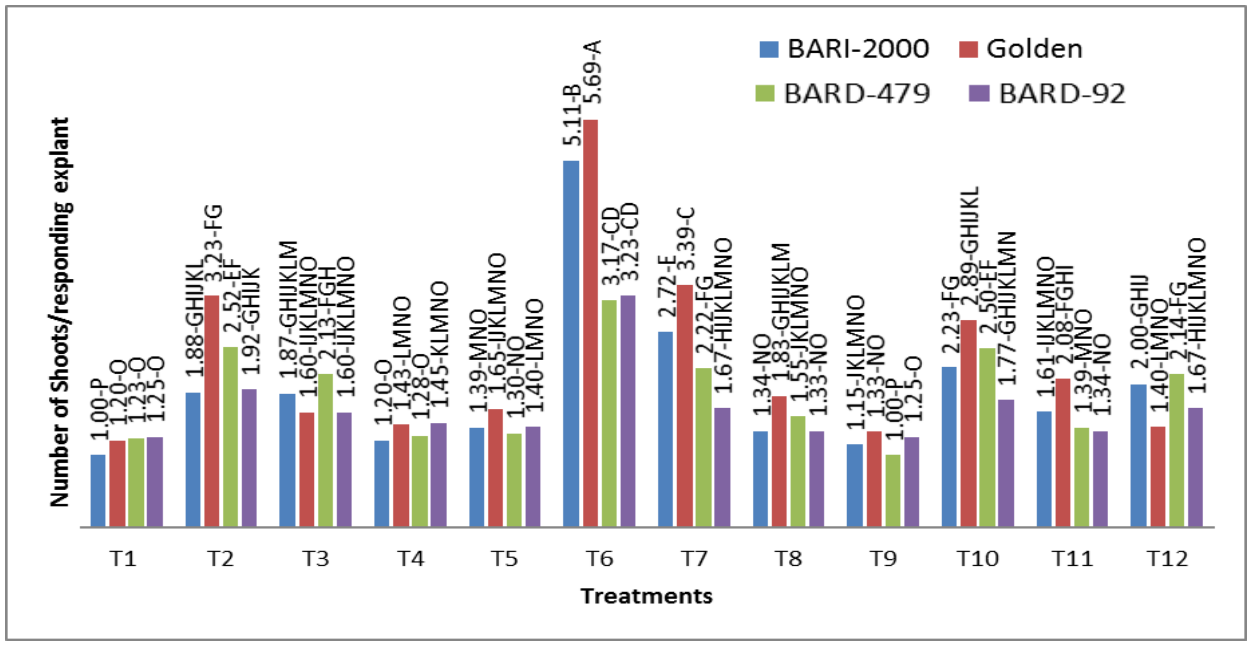

Fig. 3. Duncan's Multiple Range Test for ranking of interaction means with respect to No. of shoots/responding explants. Values sharing same letters do not differ significantly at 5% level of probability.

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Grafting

Grafting was most successfully (55%) established in Golden variety and was lowest (33.33%) in BARD-479. In our study survival rate of plants obtained from grafting was almost double as compared to that achieved by rooting of shoots. The plants grew well to maturity and produced seeds from scion and root stock parts which were distinguishable from their test colour characteristic to the variety they belonged (Figure 4). Tiwari and Tuli (2009) also reported grafting in peanut but with little success.

Fig. 4. Grafting in groundnut. **a:** Scion of Golden variety has been grafted on root stock of Chakori variety. **b:** Grafted plant after harvesting: the seed testa in Golden (scion) is red while that in Chakori (root stock) is off-white.

Venkatachalam et al. (1996) regenerated plantlets from leaf discs of peanut through organogensis using combination of BAP, Kinetin and NAA while Sarkar and Isam (2000) used only BAP and kinetin. However, results of current study coincide with those of many researchers who emphasized the use of TDZ in regeneration of plantlets from leaf explants of different *Arachis* species; *A. hypogaea* (Kanyand *et al.,* 1994) *A. correntina* (Mroginski *et al.,* 2004), *A. stenosperma* (Vijaya Laxmi and Giri, 2003) and *A. villosa* (Fontana *et al.,* 2009).

A very low concentration of TDZ (0.01 to 1.0 mg/l) is used in most of tissue culture experiments and its concentration is of immense importance in determining the pathway of plant growth (Akasaka *et al.,* 2000). It has been found that low concentration of TDZ induces multiple shoots while slightly higher concentration switches the pattern to callus induction in *Cajanus cajan* (Singh *et al.,* 2003).

The shoot bud elongated well when shifted to medium containing BAP but lacking TDZ. These results are in close agreement with those of previous findings (Gill and Ozias-Akins, 1999; Ahmad and Rahim, 2007; Fontana *et al.,* 2009). Most of cytokinins used in plant tissue culture like BAP, Zeatin and kinetin contain adenine in their structural formula while TDZ is a non-adenine type highly active cytokinin which has a wide range of effects on cultures including induction of multiple buds and inhibition of their elongation (Huetteman and Preece, 1993). This problem is overcome by shifting of buds to a medium containing adenine-type cytokinens (Fontana *et al.,* 2009).

In most of experiments the regenerated shoots are subjected to root induction followed by transplantation in soil, which results in poor survival of plants (Zhao *et al.,* 2012). The current study showed that problem could be overcome by grafting of regenerated shoots on germinated seedlings thus improving survival rate and reducing the time span significantly.

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CONFLICT OF INTEREST

All the authors have declared that no conflict of interest exists.

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