# INFLUENCE OF BAP ON *IN VITRO* REGENERATION OF SHOOTS FROM IMMATURE LEAVES OF GROUNDNUT *(ARACHIS HYPOGAEA* L.)

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**Abstract :** Cytokinins are used in *in vitro* protocols singly or in combinations with auxins to induce cell proliferation and promote shoot regeneration. We report a protocol for efficient regeneration of immature leaf explants from groundnut *(Arachis hypogaea* L.) var. Kadiri-6 and K-134 using a combination of NAA and BAP. A maximum of 90% regeneration with more than 7 shoots per explant was obtained from explants cultured on MS medium with 4 mg/L BAP and 1 mg/L NAA with subsequent substitutions of NAA with $AgNO_3$ for shoot induction and $AgNO_3$ by $GA_3$ for elongation of the shoots. The levels of BAP in the culture medium significantly influenced the frequency of regeneration. This protocol of indirect regeneration from the immature leaves may be used in genetic transformation protocols of groundnut with higher efficiency of recovery of plantlets.

**Keywords :** *Arachis hypogaea*, BAP, Immature leaves, Regeneration

## INTRODUCTION

Peanut or Groundnut *(Arachis hypogaea* L.) is one of the major oil seed crops cultivated in warm tropical and subtropical regions of the world. Groundnut cultivation suffers from several production constraints like biotic (diseases and insect pests) and abiotic stresses (drought, salinity, etc.). The naturally existing diversity of groundnut does not have sources of enough genetic resistance to be utilized in conventional breeding programmes. This necessitates the exploitation of the non conventional approaches of crop improvement like genetic transformation to create new variability and to develop cultivars with transgenic resistance. For effectively utilizing the genetic transformation system in any crop species, efficient and high frequency regeneration protocols play a key role. Legumes, in general, are treated are recalcitrant to *in vitro* manipulations. However, in groundnut earlier workers have reported regeneration protocols which are based mainly on the direct organogenesis path utilizing the de-embryonated cotyledon as explant (Radhakrishnan *et al.,* 2000). In *Agrobacterium*-mediated genetic transformation, the major approach in dicot plants, calli are more amenable for agro-infection. The indirect regeneration through callus from immature leaves as an explant is least exploited for genetic transformation of groundnut. Immature leaves from young seedlings have been used as explants to achieve organogenesis earlier in groundnut (Narsimhulu and Reddy, 1983; Baker and Wetzstein, 1992; Akasaka *et al.,* 2000). The protocols reported from most of these workers had a low frequency of regeneration with only a few number of shoots recovered. The major factors limiting indirect organogenesis include low induction rate, asynchronous shoot development and slow initial growth of plantlets.

The major advantage of immature leaf-derived organogenesis systems is the relative ease of obtaining material for explanting (Vadawale, *et al.,* 2011). MS medium containing NAA and BAP is considered as most promising combination for shoot bud formation from immature leaflet explants (Cheng *et al.,* 1992, Akasaka *et al.,* 2000). Immature leaf culture protocol and organogenesis in groundnut using culture medium supplemented with different concentrations of the auxin – NAA as sole growth regulator has been has recently been reported by Tiwari and Tuli (2009). Since the induction of shoots in response of cytokinin is a common event as it provides purine and pyramidine bases which are essential in basic physiological processes and in the rapid cell division (Victor *et al.,* 1999), it was thought appropriate to explore the influence of varying levels of BAP, the most used cytokinin in tissue culture experiments, for improving the regeneration frequency of shoots from immature leaves of groundnut.

## MATERIAL AND METHOD

### Seed Material

Two groundnut cultivars Kadiri-6 and K-134, obtained from the germplasm collection at the Directorate of Groundnut Research (DGR) were used in the study. Immature leaflets dissected out from the mature dry seeds were used as explants. The growth regulators used were 1 mg/L NAA in combination with four levels of BAP viz. 0, 1, 2, 3, 4 and 5 mg/L.

### Explant preparation

The dry seeds from stored pods were disinfested with 70% (v/v) ethanol for 1 min, followed by treating with 1% (v/v) mercuric chloride solution for

3 min under constant agitation and then washed five times with sterile distilled water. The sterilized seeds were soaked in sterile distilled water overnight. The testae of the sterilized seeds were removed aseptically and seeds were split into two halves (two cotyledons) to dissect out the immature leaves attached to the embryo. The immature leaflets were directly used as explants.

**In vitro culture and regeneration**

The culture medium used was MS medium (Murashige and Skoog, 1962) with vitamins of B5 (Gamborg *et al.,* 1968*),* containing 30 g/L sucrose and 6 g/L agar. The callus cultures were initiated on medium supplemented with the four levels of BAP viz. 0, 1, 2, 3, 4, 5 mg/L in combination with 1 mg/L NAA. Sixty explants each from the two cultivars were cultured on 25 ml solidified medium in 90 mm sterile disposable plastic petri plates. To induce shoots, explants were transferred to MS media supplemented with $AgNO_3$ in place of NAA. For elongation and expansion of the shoots, the explants with shoot buds were transferred to MS medium containing 1 mg/L $GA_3$ substituted for $AgNO_3$. All the cultures were incubated at 26±1°C and 16 hr photoperiod with 3000 lux illumination.

The fully expanded shoots were transferred to a root inducing medium containing 1 mg/L NAA as the sole growth regulator. Once the plantlets reached a height of 4-5 cm and developed at least 5-6 roots, they were floated on sterile Hoagland solution in test tubes and covered with a plastic film (Fig. 1G), and transferred to a glasshouse under controlled conditions for hardening. The plants which were actively growing in the liquid medium were later transplanted to a potting mixture in earthen pots.

Data was recorded after each transfer (two weeks) and the frequencies of responding explants were converted to percent. The data were arcsine transformed and statistically analyzed.

**RESULT AND DISCUSSION**

In the callus induction medium, explants became green and enlarged within 7 days of culture. The extent of responsive explants and the number of buds per explant differed significantly with levels of BAP in the callus initiation medium (P =0.01). The explants were found to be most responsive and had the maximum frequency of shoot bud formation in media supplemented with 4 mg/L of BAP and 1 mg/L NAA.

It was observed that supplementation with BAP alone did not produce shoot buds though the explant responded to culture initiation. Shoot proliferation was enhanced by replacing NAA with 1 mg/L $AgNO_3$ and the soot buds were elongated by subsequent transfer to the medium containing 1 mg/L $GA_3$ in place of $AgNO_3$. The highest frequency of shoot bud formation (in cv. Kadiri-6; 90%; and in cv. K-134; 86.6%) and the number of shoots per explant (Kadiri-6; 7.21±0.10 and in cv. K-134; 7.24±0.20) were obtained from the explants cultured on media supplemented with 4 mg/L BAP and 1 mg/L NAA on transfer to the shoot induction and elongation media (Table 1).

The initiation of the shoot buds in the regenerating explants was from the base of the rachis of the leaf (Fig. 1C) and finally the entire lamina developed shoot bud initials which elongated further and expanded to full shoots (Fig. 1D). Subcultures of the initial explant after separating the developed shoots back into the shoot proliferation media resulted in further production of shoots from those explants. The elongated shoots, on transfer to the rooting medium, developed roots within three weeks of incubation (Fig. 1F). The shoots which developed 4-6 roots and started growing normally in the suspension medium in test tube (Fig. 1G) on further transfer to potting mixture had grown fully and developed pods (Fig. 1H). There were no notable phenotypic difference among the regenerated shoots as they were quite like the control plants germinated from seeds under glass-house conditions.

In both the cultivars studied, there was an increase in the regeneration frequency and the maximum was at 4 mg/L of BAP; after which the regeneration frequency decreased (Fig 2). A similar retardation in response by higher concentrations of BAP was reported earlier by Verma *et al.,* (2009). However, the studies by Venkatachalam *et al.,* (2000) reported that higher concentrations of cytokinin enhanced the differentiation of shoots. Though there are earlier reports on the role of growth regulators in enhancing the frequency of regeneration from immature leaves of groundnut, the supplementation of different levels of auxin was reported to play a positive role (Tiwari and Tuli, 2009). In the present study we report varying levels of the cytokinin which enhance the frequency of regeneration. The enhancement in the regeneration may possibly due to a better auxin:cytokinin ratio used, which is a decisive factor for the regeneration of multiple shoot buds in groundnut (Banerjee *et al.,* 2007). The influence of plant growth regulators depends not only on the concentrations used but also on their interaction with the endogenous growth regulators of the plant (Roy and Banerjee, 2003). Enhancement of the regeneration of immature leaves of groundnut by combinations of growth regulators has been reported earlier also (Cheng *et al.*, 1992, Akasaka *et al.*, 2000).

Addition of $AgNO_3$, replacing the auxin after the culture initiation played a vital role in enhancing the shoot bud induction. This might be due to the modulation of the ethylene produced *in vitro* as reported earlier (Pestana *et al*., 1999). Use of $GA_3$ for enhancing the elongation of the shoots also has been profitably exploited earlier in groundnut tissue

culture (Radhakrishnan *et al.*, 2000). In the present investigation, a combination of these three treatments have resulted in a simple and efficient regeneration protocol from the immature leaves of groundnut, which can be very easily adopted for the genetic transformation protocols in groundnut.

The regenerated plantlets showed a very high rate of acclimatization (90%) and all the plants that survived grew to maturity and set pods in glasshouse. The protocol used for acclimatization of the rooted shoots in liquid medium was found to be better that directly transferring the plantlets to a potting mixture.

In conclusion, the results reaffirm the efficacy of BAP for shoot bud induction and the use of $AgNO_3$ for enhancing organogenesis and the protocol described here is a substantial improvement over the immature leaflet explant derived regeneration protocols reported earlier in groundnut.

**Table 1 :** Effect of plant growth regulator combination treatments on multiple shoot bud formation in immature leaf explant

| Growth regulator (mg/L) | | Mean frequency of explants developing shoots (%) | | Mean number of shoots per explant | |
|---|---|---|---|---|---|
| BAP | NAA | Kadiri-6 | K-134 | Kadiri-6 | K-134 |
| 0 | 1 | 48.33±1.60 | 46.67±1.36 | 0.00±0.00 | 0.00±0.00 |
| 1 | 1 | 55.00±2.10 | 58.33±0.83 | 1.52±0.02 | 1.56±0.05 |
| 2 | 1 | 60.00±1.36 | 65.00±2.10 | 3.10±0.14 | 3.36±0.10 |
| 3 | 1 | 73.33±1.36 | 73.33±1.36 | 3.84±0.18 | 5.05±0.11 |
| 4 | 1 | 86.67±1.36 | 90.00±0.96 | 7.21±0.10 | 7.24±0.20 |
| 5 | 1 | 75.00±2.50 | 80.00±1.36 | 2.81±0.16 | 3.44±0.10 |

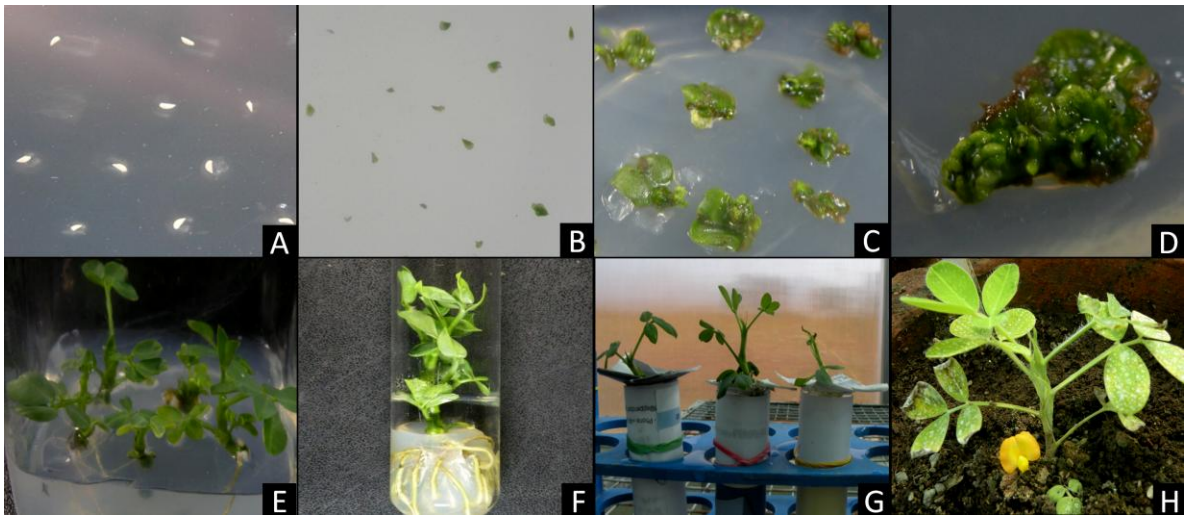


**Fig. 1.** Various stages of shoot development from immature leaf explants. (A) Immature leaf explants on culture initiation medium. (B) Green and enlarged immature leaflet. (C) Shoot bud formation on leaflet in MS medium containing 4 mg/L BAP + 1 mg/L NAA after 3 weeks. (D) Leaflet turned into a cluster of shoot buds. (E) Elongated shoot on MS medium for elongation. (F) Rooted shoot with secondary and tertiary roots on MS rooting medium. (G) Acclimatization of the plantlets in Hoagland solution. (H) Plants growing on potting mixture.

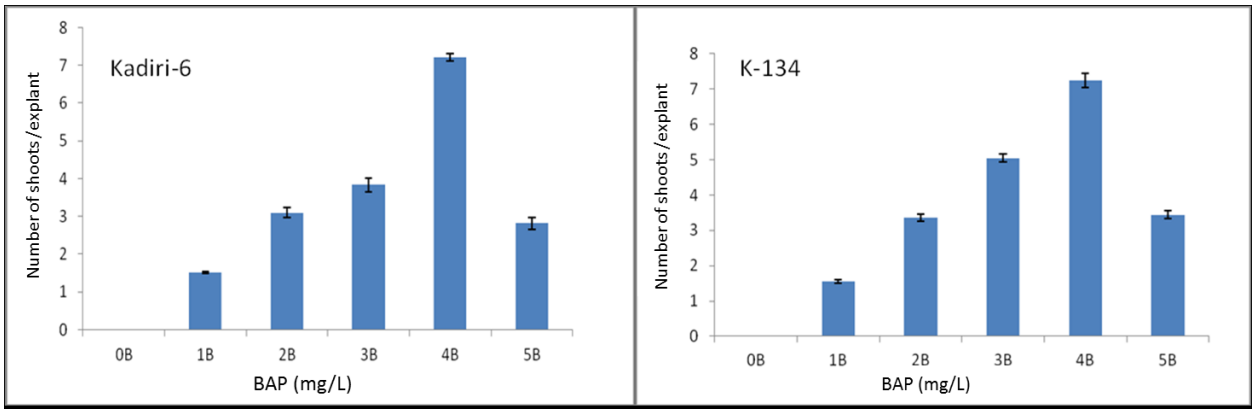


**Fig 2.** Effect of BAP on regeneration of shoots per explant in the two groundnut varieties Kadiri-6 and K-134

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