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IN VITRO MICROPROPAGATION OF BLACK GRAM (*VIGNA MUNGO* L) THROUGH SHOOT TIP EXPLANTS INDUCED BY THIDIAZURON (TDZ)

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ABSTRACT
The effect of Thidiazuron (TDZ) was investigated on *in vitro* shoot proliferation from shoot tip explants of Black gram (*Vigna mungo*.L). Murashige and Skoog (MS) medium containing TDZ (1.0–8.0 mg/L) was effective in inducing shoot buds and maintaining high rates of shoot multiplication on hormone free medium. The highest shoot regeneration frequency (78%) and mean number (06.0 ± 0.43) of shoots per explants were achieved from shoot tip explants segments cultured on MS medium supplemented with (6.0mg/L mg/L) TDZ for 4 weeks prior to transfer to MS medium without TDZ for 8 weeks. The elongated shoots were transferred to Indole Butyric Acid (IBA) (1.0mg/L–5.0 mg/L) for root induction. Rooting was observed within two weeks of culture. The regenerated shoots rooted best on MS medium containing (3.0 mg/L) indole-3-butyric acid (IBA). Rooted plantlets were successfully hardened under culture conditions and subsequently established in the field conditions.

KEYWORDS: *In Vitro* Micropropagation, Shoot Tip Explants, Black Gram (*Vigna mungo*.L).

INTRODUCTION

Legumes, broadly defined by their unusual flower structure, podded fruit, and the ability of 88% of the species examined to date to form nodules with rhizobia (De Faria *et al.*, 1989), are second only to the Poaceae in their importance to humans. The 670 to 750 genera and 18,000 to 19,000 species of legumes include important grain, pasture. Legumes should be planted in light soils, not so much for their own crops as for the good they do to subsequent crops. Leguminous plants are quite different to regenerate *in vitro.* Grain legumes represent one of the most valuable sources of proteins for human and animal nutrition and are also responsible for nitrogen enrichment of soil through symbiosis with Rhizobium. Legumes are largely cultivated in the Mediterranean basin, Middle East, Asia and South America and to date, they have been qualitatively and quantitatively improved by conventional breeding. However, the lack of resistance to several pest diseases still remains the major cause of significant loss of edible product. Recent advances in genetic engineering have clearly demonstrated the possibility of incorporating foreign genes for desired agronomic traits while preserving the existing characteristics of improved genotypes.

Blackgram (*Vigna mungo* L. Hepper) is an important grain legume, reported to have originated in India (Dhuri *et .al.*, 1986). In India, the protein requirement of the vegetarian population and those who cannot afford meat is met through legumes. *Vigna mungo* is also used as nutritive fodder for cattle or as a green manure. India is the largest producer accounting for more than two-thirds of the world's total production of black gram (Jeswani and Baldev 1990). The crop is also widely grown in other Asian countries, Africa, and Australia.

In India, the total area under black gram cultivation is 3.01 million ha with a production of 1.2 metric tons but the yield per hectare is stagnant though there is an increase in land under cultivation. The production constraints are susceptibility to yellow mosaic virus (VMYMCV) (Sahoo *et .al.*, 2002), fungal pathogens (powdery mildew, Cercospora leaf spot), bruchids (Sahoo *et .al.*, 2002), and pod borer (Rao and Chand 2006; Sarma and Borah 2004; Satyanarayana *et .al.*, 1990). Blackgram has been found to be recalcitrant to *in vitro* regeneration (Sahoo *et .al.*, 2002) and efficiency of the multiple shoot formation and regeneration has been found to be dependent on various parameters, *viz.* explant size, age, type and genotype, and media composition (Saini and Jaiwal 2002). Moreover, the above protocols are restricted to a few cultivars and none of these systems were found to be suitable for routine genetic transformation of blackgram.

The most critical events affecting cotyledonary nodebased, *Agrobacterium*-mediated transformation are induction of shoots from the wounded site and the high frequency of multiple shoots per explant. Genetic transformation of blackgram has been reported using

cotyledonary nodes (Bhomkar *et .al.,* 2008; Saini *et .al.,* 2003) and shoots tips (Saini and Jaiwal 2005). However, in using cotyledonary node explants, only 21 (Saini and Jaiwal 2003) to 40% (Bhomkar *et .al.,* 2008) of the recovered shoots carried the gene of interest, whereas for shoot tip explants only 19% (Saini and Jaiwal 2005) of the total shoots recovered were transformed with the transgene. Moreover, the frequency of transgene transmission to the next generation was also poor when compared with other legumes (Popelka *et .al.,* 2006; Sarmah *et .al.,* 2004). The major factor affecting blackgram transformation is the non-availability of a suitable regeneration and transformation system in blackgram.

In the above-published protocols, *in vitro* regeneration of blackgram was achieved by using the cytokinin group of growth hormones, BAP, or Kinetin. Amongst the cytokinins, thiadiazuron (TDZ) was also found most effective for shoot proliferation in tissue culture (Huetteman and Preece 1993) and was also found to be superior than other cytokinins (Thomas 2003; Thomas and Puthur 2004) for induction of multiple shoot buds.

Though some micro propagation studies have been conducted so far, this paper deals with the efficient plant regeneration system with large number of shoots within a short period from shoot tip explants of Black gram (*V. mungo*.L).

Materials and Methods: Plant material for *in vitro* regeneration: Seeds of *V. mungo* cultivars Co-5 were obtained from ICRISAT Hyderabad Telangana Seeds were also collected from the local market and included in the present investigation. Seeds were stored in plastic containers after application of fungicides (Bavisthin) at the rate of 2.0 g per kg seeds.

Surface sterilization of seeds of *V. mungo:* The seeds were washed thoroughly in tap water 3–5 times and placed in 1% (v/v) Teepol solution (Reckitt Benckiser, India) which was kept under running tap water for 15 min. Then the seeds were disinfected with 0.1% (w/v) Mercuric chloride ($HgCl_2$) for 5 min. Finally the seeds were rinsed 3–4 times in sterile distilled water and inoculated on moist cotton in sterile test tubes. To assure uniform and rapid germination of seeds, test tubes were placed in dark at 28°C for 24–48 h. Then the germinated seeds were transferred to light intensity (15 µmol/s2/s), 16 h light per day photoperiod for another 4–7 days and maintained at 25 ± 2°C and 55–60% relative humidity.

Selection of explants: Shoot tip with one or two leaf primordia, of 15-d old *in vitro* raised seedlings was selected as explants for direct shoot multiplication. The shoot tip, segments of 5–8 mm in length was excised aseptically.

Culture media and culture conditions

MS media containing 3.0% sucrose and supplemented with various concentrations cytokinin such as TDZ (1.0 – 8.0 mg/L) were used. The initial pH of the culture media was adjusted to 5.8 before addition of 0.8% (w/v) agar-agar. The medium was dispensed into culture tubes (25 ±150 mm) each containing 15 ml of the culture medium capable with non-absorbent cotton and was autoclaved at 121^0 C for 15 minutes. In each cultures tube one shoot tip explants was implanted. The cultures were maintained under 16h light provided with white fluorescent tubes (40 µ mol m-2s-2) at 25 ± 2^o C.

RESULTS AND DISCUSSION

Data on multiple shoot induction from shoot tip explants cultured on MS medium fortified with different concentrations of TDZ alone is presented in (Table-1). The important part of the present study was the preparation of contamination free explants. This was achieved by using *in vitro* germinated seedlings as an explant source. Sterilization of seeds required 0.1% (w/v) $HgCl_2$ 5 min treatment for maximum germination (98%) and minimum contamination (Narashimhulu and Reddy, 1983). A similar observation was also reported in *Vigna aconitifolia*, confirming the view that the pretreatment of seeds with specific surface sterilizing agents would predetermine the regenerating behavior of explant tissues (Godbole, *et al.*, 1984). The use of direct and large sized explants had higher survival and growth rates than the smaller ones (Hu Wang, 1983).

Table. 1: Effect of Cytokinin (TDZ) on plantlet regeneration of Black gram (*Vigna mungo*.L) from Shoot tip explants.

Hormone cone (mg/L)	% of cultures responding	Average number of shoots/ explants (S.E)*	Shoot length Mean ± SD
1.0	42	2.0 ± 0.23	1.4 ± 0.35
2.0	48	3.2 ± 0.45	1.3 ± 0.24
3.0	55	4.0 ± 0.34	1.2 ± 0.34
4.0	60	4.3 ± 0.25	1.4 ± 0.32
5.0	62	5.0 ± 0.35	2.6 ± 0.24
6.0	72	6.0 ± 0.43	2.1 ± 0.35
7.0	78	4.0 ± 0.42	2.7 ± 0.43
8.0	60	3.0 ± 0.42	1.7 ± 0.42
9.0	50	2.8 ± 0.43	1.5 ± 0.23
10.0	45	1.7 ± 0.42	1.2 ± 0.42

*** Mean ± Standard error.**

Table. 2: Rooting ability of regenerated shoots from Shoot tip explants of Black gram (*Vigna mungo*.L) cultured on MS medium supplemented with IBA.

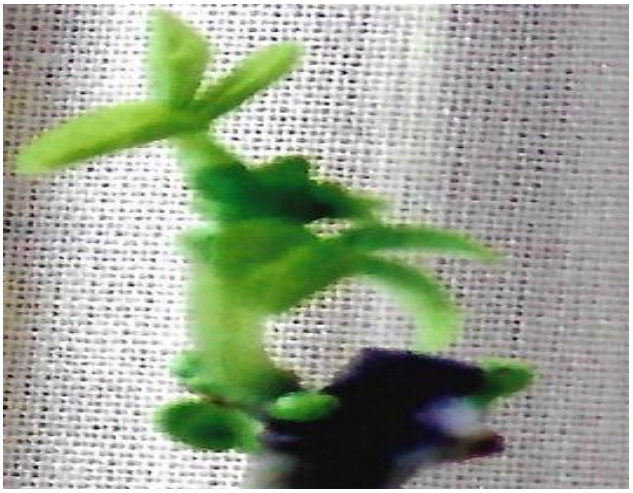
Hormone cone (mg/L)	% of cultures responding	Shoot no. Mean ± SD	Shoot length Mean ± SD
1.0	70	1.0 ±0.53	0.6 ±0.04
1.5	75	2.5 ±0.56	0.8 ±0.42
2.0	80	2.6 ±0.35	2.0 ±0.35
2.5	86	3.2 ±0.53	2.6 ±0.32
3.0	90	4.0 ±0.24	3.0 ±0.32
3.5	58	3.8 ±0.56	3.2 ±0.56
4.0	50	4.2 ±0.46	2.6 ±0.42
4.5	47	3.2 ±0.56	1.5 ±0.56
5.0	40	2.0 ±0.23	0.5 ±0.35

***Mean ± Standard Error.**

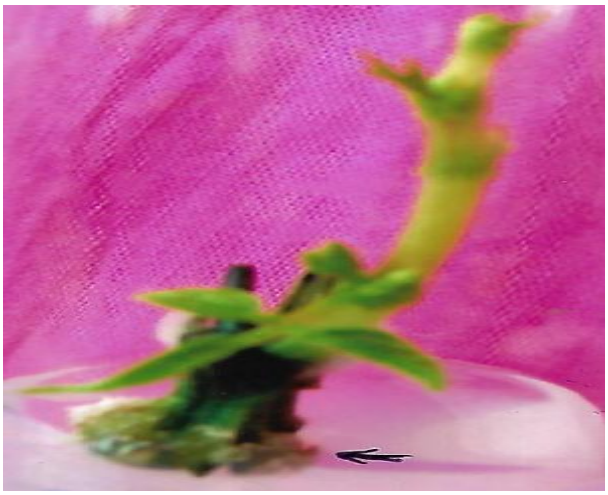
Effect of TDZ: The meristem containing explants shoot tip were excised from the surface sterilized, *in vitro* grown, 30-d old seedlings and cultured on MS medium augmented with TDZ (1.0–8.0 mg/L) for multiple shoot induction of all the different concentrations of TDZ tested, (2.0 mg/L) TDZ was found to be more effective in inducing (3.2 ± 0.45 shoot buds/explants) (Fig -1). But at high concentration of TDZ (6.0 mg/L) considerably the number of shoot induction was found to be reduced. As the concentration of TDZ was increased up to 2.0mg/L the multiple shoots number was increased but as the concentration of TDZ (4.0mg/L) to (8.0 mg/L) TDZ resulted the number of shoots were reduced. (Fig c).

The mode of action of TDZ on plant tissue during *in vitro* regeneration is not yet understood but there is a suggestion that TDZ increases the biosynthesis as well as accumulation of endogenous purine and cytokinin leading to shoot differentiation (Thomas and Katterman 1986). Since 1988, TDZ has been reported to induce adventitious shoot buds in a number of plant species (Briggs *et .al.,* 1998; Henny and Fooshee 1990). However, it was observed that mean number of multiple shoots per explant decreased with the increase (up to 3 µM) in TDZ concentration and longer duration of exposure of the explants to TDZ. Similar disadvantages, including stunted shoots, abnormal leaf morphology, etc., were reported when TDZ was used in the medium for longer duration (Jayanand *et .al.,* 2003). Therefore, explants of blackgram were exposed to TDZ for only one sub-culturing cycle comprised of 14 days. Amongst the various concentration of TDZ, 2 µM of TDZ in combination with BAP (2 µM), KIN (2 µM), and NAA (0.5 µM) was found best for induction of multiple shoots.

Rooting of Micro-shoots: The shoots raised *in vitro* (2-3 cm long) were cultured on MS medium supplemented with various concentration of IBA (1.0 – 5.0 mg/L) for the induction of roots (Table-2). High percentage of rooting ability with several roots was observed at (2.0 and 3.0 mg/L) IBA, longer roots were also observed at (3.0 mg/L) IBA. The percentage of response was increased gradually from (1.0 to 3.0 mg/L) IBA. Maximum numbers of roots were induced at (3.0 mg/L) IBA. The number of roots increased from (1.0 – 3.0 mg/L) IBA roots number was decreased from (3.0 to 5.0 mg/L) IBA. (Fig-1).



a



b

c **d**

Figure. 1: *In Vitro* Micropropagation of Black gram through Shoot tip explants a)*In Vitro* Plantlets on MS+(6.0 mg/L)) TDZ b) Multiple shoots on MS+(5.0 mg/L) TDZ c) *In Vitro* rooting from Micro shoots on MS+3.0 (mg/L) IBA after six week e) Hardening of plantlets.

Hardening of well-rooted plantlets

The elongated shoots were transferred to MS medium augmented with IBA (1.0–5.0mg/L) for root induction. Rooting was observed within two weeks of culture. Well-rooted plantlets were isolated and washed in running tap water. Later they were transplanted into plastic cups containing sterile sand and soil mixture (3:1) for hardening purposes. The well-grown plants were transferred to larger pots containing soil mixture and maintained in the field conditions. Plants grown in the field were further observed for growth and survival.

The result of present investigation show that the shoot tip explants from mature plants of *V. mungo* cultivars Co-5 could be induced to produce multiple shoots *in vitro* maximum number of shoots was induced on MS medium fortified with various concentrations of TDZ. In recent years, shoot tip explants have been preferred to produce large number of genetically identical clones (Bajaj Dhanju, 1979). Multiple shoot formation from shoot apices was obtained on MS medium supplemented with 20µM BA, 0.1µM NAA in pea (Griga *et al.*, 1986). MS-solid medium fortified with TDZ and Kn alone and in combination increased the regeneration potential of shoot apical meristems of soybean, cowpea, peanut, chickpea and bean (Kartha *et al.*, 1981). It was reported that BAP was proved to be an ideal hormone for shoot multiplication of shoot tip culture in grain legumes (Sounder Raj *et al.*, 1989).These results are also in agreement with those on *Tectona grandis* (Gupta *et al.*, :1980) *Abizzia lebbeck* (Gharyl and Maheshwari 1982) multiple shoot induction was also observed in *Ziziphus manritiana* (Sudharshan *et al.*, 2000) and *Vanilla plantifolia* (Geetha *et al.*, 2000) shoot tips cultured on MS ±cytokinin alone as it was observed in the present studies. Nasir *et al*., (1997) has studied the shoot meristem culture in 16 cultures of cotton using several media formation. They observed the best shoot developmentation MS media containing TDZ alone compared to other media with NAA / IAA in combination with TDZ. These results are to the present observation in *V. mungo* cultivars Co-5 which contain with cytokinins showed the increased number of shoots/ explants have also observed the similar results when they have cultured the shoot tips of F1 hybrids of *Paulownia*.

The capacity of shoot bud differentiation and shoot proliferation from shoot tip explants of *V. mungo* cultivars Co-5 depended on hormonal variation. There was good shoot bud induction and proliferation response only in the presence of cytokinin and no response in the basal medium. Similar results are well documented in several medicinal plants (Pattnaik and Chand 1996), *Emblila officinale* (Verma and Kant: 1996) and *Withania somnifera* (Deka *et al.*, 1999). From our study it was clear that (2.0 mg/L) BAP and Kn were significantly more effective for inducing shoot organogenesis. Well-developed shoot lets from our experimental data, it is evident that TDZ are the best suited for inducing multiple shoots In conclusion, this communication describes an efficient rapid propagation system of *V. mungo* cultivars Co-5.

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