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Effect of cytokinins and their concentration regimes on multiple shoot induction using cotyledonary node with cotyledons and embryo discs with half cotyledon explants in pigeonpea (*Cajanus cajan* L. Mill sp.)

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Abstract

Pigeonpea (*Cajanus cajan* L. Milli sp.) belongs to family Fabaceae, ranks sixth among legumes in production, which is constrained by several biotic and abiotic stresses. Three cytokinins *viz*., BAP, TDZ and zeatin and their concentration regimes were tested for multiple shoot bud induction in ICPL87119 and BSMR736 pigeonpea genotypes. Fortification of growth medium with 2.0 mg/l BAP, 4.0 mg/l TDZ and 2.0 mg/l zeatin, separately, could induce maximum number of shoot buds, 53.7, 46.1 and 40.9 respectively, per explants in both, cotyledonary node with cotyledons and embryo discs with half cotyledon explants. Any further increase in tested cytokinin concentration levels imparted reduction in multiple shoot bud induction. There was no significant effect of MS nutrient strength on root induction, MS with 0.5 mg/l IBA was most suitable for healthy and number of root induction (4.8±0.7). Reported *in vitro* method of plantlet regeneration may be exploited for genetic engineering programs for multiple stress tolerance in pigeonpea.

Key words: Cytokinin, IBA, pigeonpea, induction, regeneration, plantlet

Introduction

Pigeonpea (*Cajanus cajan* L. Milli sp.) belongs to family Fabaceae, cultivated in more than 25 countries of the world including Indian subcontinent, Africa and Central America. It ranks sixth among grain legumes in production and grown on 4.7 million hectares of agriculture land in world with the production of around 3.69 million tons annually (http://faostat.fao.org). Pigeonpea is consumed as a staple food, green vegetable and improves soil fertility through nitrogen fixation [1]. It occupies an important position in human diet as a protein source (21%) in the vegetarian population [2]. India, alone contributes more than 80% of total world pigeonpea production [3].

Pigeonpea production and productivity are constrained by several biotic and abiotic stresses, whose levels of resistance in world germplasm accessions are low to moderate. Attempts to develop pigeonpea cultivars resistant to biotic stresses, such as resistance to legume pod borer (*Helicoverpa armigera*) and *Fusarium* wilt by conventional breeding methods have shown limited success due to narrow genetic variability among the germplasm accessions. Breeding incompatibility problems associated with wild species warrant the exploration of alternative approaches. Genetic engineering technology plays a significant role as an additional tool for the introduction of agronomically useful traits in a high yielding background.

There are very few reports on *in vitro* regeneration of pigeonpea through organogenesis from unorganised callus [4]. Many independent studies have reported the multiple shoot production and plantlet regeneration through organogenesis from different explants *viz*., cotyledons, embryonic axes, cotyledonary node from mature seeds and seedling petioles [5-6]. The attempts have been made to initiate *in vitro* culture from different tissue sources in pigeonpea [7]. However, the *in vitro* regeneration protocols further need to be fine-tuned

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and improvised to achieve high frequency regeneration of plantlets. In this contest, the enhancement in multiple shoot bud induction by use of cytokinins in nutrient medium expected to be one of the potential approaches.

Cytokinins, as a hormone, are associated with cell division, modification of apical dominance, shoot differentiation *etc* and incorporated in tissue culture media for cell division and differentiation of adventitious shoots from callus and organs. The commonly used cytokinins for plant tissue culture includes benzylamino purine (BAP), isopentenyl-adenine (2-ip), furfurylamino purine (kinetin), thidiazuron (TDZ) and zeatin. The effect of cytokinins such as, BAP, kinetin and their varied levels of concentrations on multiple shoot induction has been studied in pigeonpea [7]. In another study, frequency of multiple shoot bud induction has been titrated using different cytokinins *viz*., BAP, kinetin, TDZ in eleven Indian cultivars of pigeonpea using leaf as explant [8].

Independent studies have reported that the type of explant, genotype and concentrations of cytokinin can influence the frequency of shoot bud regeneration [7-8]. The present study aims at improvisation of multiple shoot induction frequency and plantlet regeneration in pigeonpea using cotyledonary node with cotyledons and embryo discs with half cotyledon explants. Two pigeonpea genotypes *viz*., ICPL 87119 (Asha) and BSMR 736 were tested for their response to different cytokinins such as, BAP, TDZ and Zeatin, most commonly used cytokinins for organogenesis. The study identified the best concentration level of cytokinin in medium for highest number of shoot bud inductions. As per our understanding, this is the first report indicating highest number of shoot bud inductions (up to 53 shoots per explant) in pigeonpea. The medium fortified with identified concentration levels of respective cytokinins can be used for crop improvement programmes involving production of transgenic pigeonpea with improved transformation efficiency by formation of more number of shoots per explant co-cultivated.

Materials and methods

Plant material

We used two pigeonpea genotypes *viz*., ICPL 87119 (Asha), which is moderately resistant to *Fusarium* wilt, sterility mosaic disease and BSMR 736, which is sterility mosaic disease resistant genotype for present experiment. Seeds of pigeonpea cultivars ICPL 87119 (Asha) and BSMR 736 were obtained from Agriculture Research Station (ARS), Gulburga. Healthy seeds with uniform size, shape and colour were surface sterilized with 70% ethanol treatment for 1 min followed by two rinses with sterile distilled water for 2 min each. Further, seeds were subjected to 0.1% (W/V) aqueous mercuric chloride solution treatment for 5 min followed by two rinses with sterile distilled water for 2 min each. The surface sterilized seeds were used for inoculation on Murashige and Skoog's (MS) medium having different cytokinins and their consecration regimes.

Culture media and conditions

The culture medium was that of MS with 3% (W/V) sucrose. For multiple shoot bud induction, the culture medium was augmented with 1, 2, 3, 4, 6, 8 and 10 mg/l BAP, TDZ and zeatin, separately. The pH of the medium was adjusted to 5.8 prior to adding 8 gm/l agar-agar. Initially, MS medium was sterilized at 121°C for 15 min and filter sterilized growth hormones were added. Cultures were maintained at 25±2°C under light intensity of 1000 lux with 16 hr/day photoperiod.

Explant preparation and effect of cytokinins on shoot bud induction

The experiment was designed to compare the effect of three cytokinins *viz*., BAP, TDZ and zeatin with their concentration regimes (1, 2, 3, 4, 6, 8 and 10 mg/l) on shoot bud induction. The explants used for study were cotyledonary node with cotyledons and embryo discs with half cotyledon. For cotyledonary node with cotyledons as explants, the surface sterilized seeds were inoculated on basal MS supplemented with increasing concentrations of growth hormones. And in case of embryo discs with half cotyledon as explants, the surface sterilized seeds were soaked overnight in sterile distilled water; seed coat along with half cotyledon was detached using sterile forceps retaining embryo discs with other half part of cotyledon. After 8-10 days of culture, the number of explants forming shoot buds and shoot buds per explant were counted. The cotyledonary node with induced multiple shoot buds were sub-cultured on the MS medium with respective growth hormone augmentations for shoot bud elongation up to 10-12 days. Experiment was repeated twice using a total of 50 explants for each treatment. The growth hormone solutions were prepared *viz*., BAP in 1N NaOH; TDZ in DMSO and Zeatin in 1N NaOH, and filter sterilized before using in culture medium. The explants cultured on MS medium devoid of growth hormone fortification were maintained

as experimental controls and the effect of different cytokinins and their concentration regimes was analyzed.

Rooting of elongated shoots

The treatments maintained for rooting were culture medium MS + (0-2 mg/l) IBA; ½ MS and ½ MS + 0.50 mg/l IBA. A set of fifty shoots per rooting medium treatment were maintained for this experiment and the experiment was repeated twice. After 10-15 days of incubation in rooting media, individual shoots was observed for root induction and the number of roots induced per shoot.

Statistical analysis

All the experiments were repeated two times and standard deviation was calculated. The experimental design used for statistical analysis of shoot bud induction and rooting was completely randomized block design and means were evaluated at 5% level of significance using Duncan's multiple range test. Statistical t-test was performed to test the response of pigeonpea genotypes and explant types used to different experimental treatments.

Results and discussion

The surface sterilized seeds, cultured on MS basal and MS medium supplemented with different cytokinins and their concentration regimes indicated 50-60% germination after 7 days in both genotypes (ICPL 87119 and BSMR 736).

Multiple shoot bud induction in response to BAP concentration regimes

The number of explants *i.e.* cotyledonary node with cotyledon explants responded to different concentration regimes of BAP for shoot bud induction was ranged from 0.0 to 36.0 in case of ICPL 87119 and from 0.0 to 36.5 in case of BSMR 736 (Table 1). The maximum number of explants responded for shoot bud induction was noticed at 2.0 mg/l BAP concentration level, whereas it was list at 10 mg/l BAP, in both genotypes. The observation of number of shoot bud induced per explant was ranged from 0 to 52.6 for ICPL 87119 and from 0 to 53.7 for BSMR 736 (Table 2). The maximum number of shoot buds induced was recorded in MS medium supplemented with 2.0 mg/l BAP (Fig. 1). For embryo disc with half cotyledon explants, the explants response ranged from 0 to 26.5 (ICPL 87119) and 0 to 27.5 (BSMR 736) (Table 3). The number of explants responded to BAP fortification was at its maximum when medium was fortified with 2.0 mg/l BAP. Interestingly, there was a concomitant decrease in explants response with increase in BAP concentration. Similar kind of explants response to multiple shoot bud induction was noticed in both test pigeonpea genotypes. The number of shoot bud induced from embryo discs with half cotyledon explants was ranged from 0 to 4.8 in ICPL 87119, whereas it was from 0 to 4.4 in BSMR 736 (Table 2). In MS medium supplemented with 2.0 mg/l BAP recorded highest number shoot buds (4.8; ICPL 87119) when embryo discs with half cotyledon was used as explant.

The behavioral response of two test genotypes to same BAP level was non-significant (p>0.72) as

Table 1. Effect of BAP, TDZ and zeatin on direct multiple shoot induction from cotyledonary node with cotyledons explant of pigeonpea genotypes, ICPL 87119 and BSMR 736, after 10 days of culture (50 explants)

Growth hormone concentrations	Number of explants responded (mean ±SD)					
	BAP		TDZ		Zeatin	
	ICPL 87119	BSMR 736	ICPL 87119	BSMR 736	ICPL 87119	BSMR 736
0 mg/l	0.0±0.0 ^{c}	0.0±0.0^{d}	0.0±0.0^{e}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{c}
1 mg/l	23.5±2.1^{b}	21.0±1.4^{b}	22.5±3.5^{c}	23.5±2.1^{bc}	29.5±2.1^{a}	30.5±3.5^{a}
2 mg/l	36.0±1.4^{a}	36.5±2.1^{a}	23.5±2.1^{c}	23.5±3.5^{bc}	33.0±1.4^{a}	32.0±1.4^{a}
3 mg/l	32.0±2.8^{a}	32.5±2.1^{a}	29.5±2.1^{ab}	29.0±1.4^{ab}	22.5±3.5^{b}	22.0±1.4^{b}
4 mg/l	26.0±2.8^{b}	24.5±2.4^{b}	33.5±3.5^{a}	33.5±2.1^{a}	21.5±3.5^{b}	21.5±2.1^{b}
6 mg/l	24.0±1.4 ^{b}	24.5±0.7^{b}	25.5±2.1^{bc}	24.5±3.5^{bc}	21.5±2.1^{b}	20.5±1.2^{b}
8 mg/l	22.5±2.1 ^{b}	21.5±1.4^{b}	23.5±2.2^{c}	22.5±3.5^{bc}	19.0±2.8^{b}	19.0±1.8^{b}
10 mg/l	21.0±1.4 ^{b}	16.0±2.8^{c}	17.5±3.5^{d}	18.5±2.9^{c}	18.5±2.1^{b}	17.5±3.5^{b}

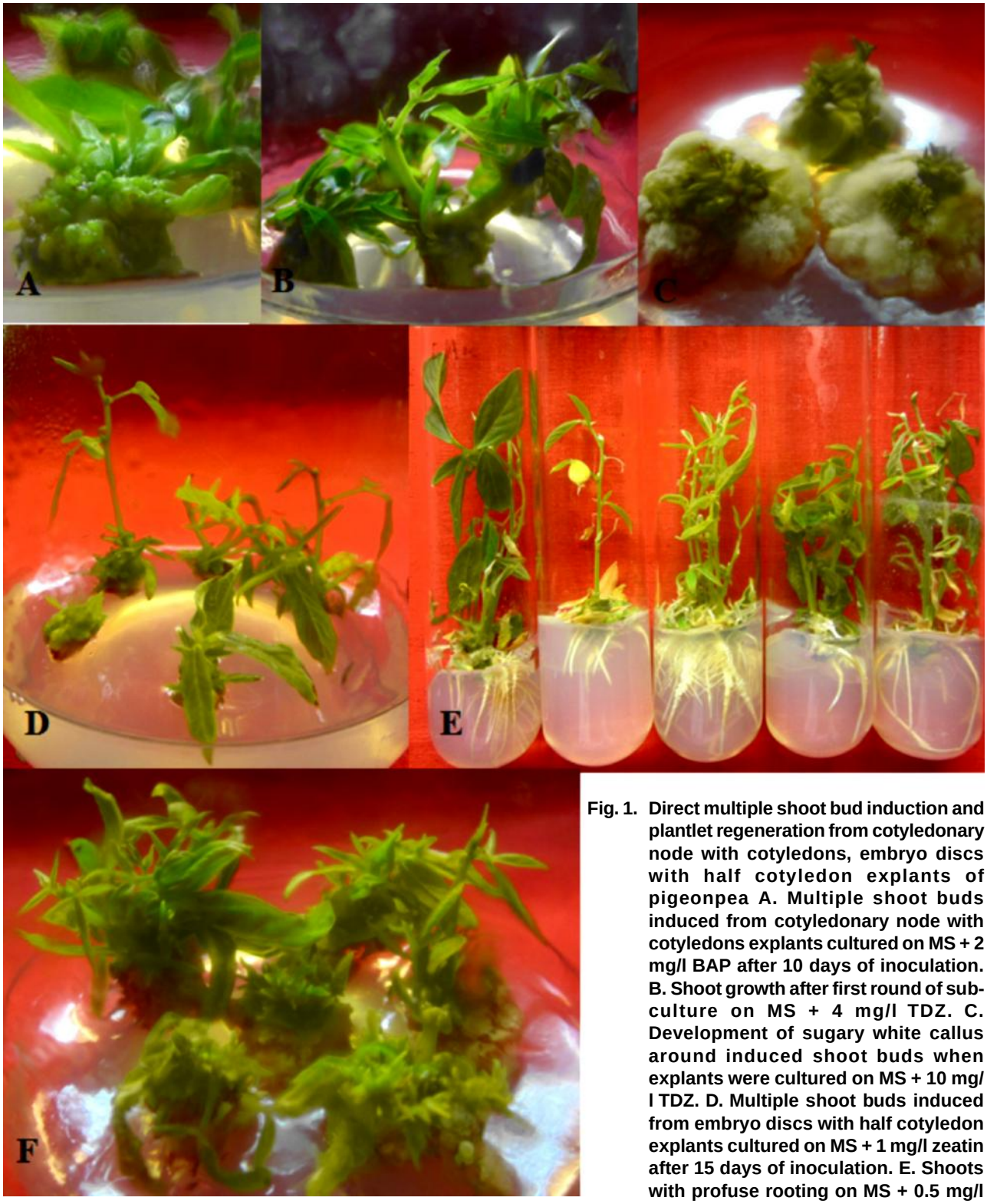

Fig. 1. Direct multiple shoot bud induction and plantlet regeneration from cotyledonary node with cotyledons, embryo discs with half cotyledon explants of pigeonpea A. Multiple shoot buds induced from cotyledonary node with cotyledons explants cultured on MS + 2 mg/l BAP after 10 days of inoculation. B. Shoot growth after first round of sub-culture on MS + 4 mg/l TDZ. C. Development of sugary white callus around induced shoot buds when explants were cultured on MS + 10 mg/l TDZ. D. Multiple shoot buds induced from embryo discs with half cotyledon explants cultured on MS + 1 mg/l zeatin after 15 days of inoculation. E. Shoots with profuse rooting on MS + 0.5 mg/l IBA. F. Multiple shoot buds induced (up to 52 shoot buds) from cotyledonary node with cotyledons explants cultured on MS + 2 mg/l BAP after 14 days of inoculation

revealed by t-test statistical analysis. At the same time, there was a significant difference between the two explant types (cotyledonary node with cotyledons and embryo discs with half cotyledon) for their response to same BAP concentration regime (p<0.05).

Table 2. Number of shoot bud induced in response to different concentration regimes of cytokinins (BAP, TDZ, zeatin) from cotyledonary node with cotyledons and embryo discs with half cotyledons explants

Growth hormone concentrations	Number of shoot bud induced per explant (mean ±SD)											
	Cotyledonary node with cotyledons						Embryo discs with half cotyledon					
	BAP		TDZ		Zeatin		BAP		TDZ		Zeatin	
	ICPL 87119	BSMR 736	ICPL 87119	BSMR 736	ICPL 87119	BSMR 736	ICPL 87119	BSMR 736	ICPL 87119	BSMR 736	ICPL 87119	BSMR 736
0 mg/l	0.0±0.0^{f}	0.0±0.0^{f}	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{d}
1 mg/l	29.9±5.6^{c}	30.2±6.2^{c}	22.8±4.6^{d}	26.0±3.9^{d}	35.9±4.9^{b}	35.3±6.0^{b}	2.8±0.6^{cd}	3.1±0.6^{bc}	2.5±0.5^{c}	2.8±0.6^{c}	4.2±1.0^{a}	4.2±0.8^{a}
2 mg/l	52.6±5.9^{a}	53.7±4.9^{a}	30.5±3.9^{bc}	32.2±3.3^{bc}	40.4±4.5^{a}	40.9±5.8^{a}	4.8±0.8^{a}	4.4±0.5^{a}	3.1±0.7^{c}	3.3±0.7^{bc}	3.5±0.5^{b}	3.6±0.5^{b}
3 mg/l	40.4±5.8^{b}	41.2±4.9^{b}	31.9±4.3^{b}	34.9±2.6^{b}	30.8±3.0^{c}	29.3±3.9^{c}	3.5±0.5^{b}	3.2±0.6^{b}	4.0±0.9^{b}	3.9±1.1^{ab}	2.8±0.8^{c}	2.9±0.7^{c}
4 mg/l	32.9±5.2^{c}	33.8±4.2^{c}	41.1±5.7^{a}	46.1±5.3^{a}	27.3±4.4^{c}	25.3±3.8^{d}	3.0±.0.7^{bc}	2.7±0.7^{cd}	4.7±1.0^{a}	4.5±0.8^{a}	2.7±0.7^{c}	2.6±0.5^{c}
6 mg/l	23.9±4.2^{d}	25.9±1.7^{d}	31.4±2.9^{bc}	33.4±3.0^{b}	22.4±3.3^{d}	23.5±3.3^{d}	2.5±0.5^{cd}	2.6±0.5^{d}	2.7±0.7^{c}	2.9±0.6^{c}	2.5±0.5^{c}	2.4±0.5^{c}
8 mg/l	19.7±4.3^{de}	21.2±3.7^{e}	27.6±3.7^{c}	29.6±4.5^{c}	20.0±3.8^{d}	21.7±3.3^{d}	2.4±0.5^{d}	2.6±0.5^{d}	2.7±0.7^{c}	2.7±0.5^{c}	2.4±0.5^{c}	2.5±0.5^{c}
10 mg/l	18.7±2.8^{e}	20.1±3.4^{e}	22.8±2.7^{d}	25.8±3.1^{d}	21.0±3.0^{d}	22.0±2.5^{d}	2.3±0.5^{d}	2.5±0.5^{d}	2.5±0.5^{c}	2.6±0.5^{c}	2.6±0.5^{c}	2.4±0.5^{c}

Similarly, there was no significant difference for number of shoot bud induced per explants in response to same BAP level between two test genotypes (p>0.82). Whereas, the effect of same BAP level on shoot bud induction from cotyledonary node with cotyledons and embryo discs with half cotyledon was found be significant (p<0.001). The analysis of means indicated that the two treatments, 2.0 mg/l and 3.0 mg/l BAP, were on par for number of explants responded to BAP fortification (Table 1). On other hand, the treatment with 2.0 mg/l BAP level was superior over other treatments for number of shoot bud induced per explant (Table 2). Interestingly, there was a concomitant decrease in resonance of explants for shoot bud induction with further increase in BAP levels in growth medium (Figs. 2 and 3). The similar kind of response by explants to increasing concentrations of cytokinins has been documented by Geetha *et al.* (1998). The effect of two cytokinins *viz*., BAP and Kinetin, and their concentration regimes have been studied for shoot bud induction from cotyledonary node, epicotyl, hypocotyl, cotyledon and leaf explants; reported that the 2.0 mg/l BAP level could potentially induced maximum number of shoot buds from all test explants [7].

Multiple shoot bud induction in response to TDZ concentration regimes

The number of cotyledonary node with cotyledon explants responded to different TDZ concentration regimes ranged from 0 to 33.5 in case of both test genotypes (Table 1). Further, in both genotypes, maximum number of explants responded for shoot bud induction was noticed at 4.0 mg/l TDZ concentration level. It was observed that the number of shoot bud induced per explant was maximum at 4.0 mg/l TDZ concentration and it ranged from 0 to 41.1 (ICPL 87119) and from 0 to 46.1 (BSMR 736) in test genotypes (Table 2). The embryo disc with half cotyledon explants response to increasing TDZ concentration regimes ranged from 0 to 22.0 in ICPL 87119 and 0 to 25.0 in BSMR 736. The maximum number of explants responding was recorded in BSMR 736 at 4.0 mg/l TDZ concentration regime (Table 3). The number of shoot bud induced from embryo discs with half cotyledon explant was ranged from 0 to 4.7 (ICPL 87119) and from 0 to 4.5 (BSMR 736) (Table 2). At TDZ concentration regime of 4.0 mg/l, the highest number shoot buds (4.7) was recorded in ICPL 87119 when embryo discs with half cotyledon was used as explant.

There was no significant difference between the response of two test genotypes to different TDZ concentration levels (p>0.43). On other hand, the effect of TDZ concentration regime on two test explants response found significant (p<0.001). The statistical analysis revealed that there was no significant difference between two test genotypes for number of shoot bud induced per explant in response to same TDZ level (p>0.83). At the same time, the two test explants showed significant difference for their response to multiple shoot bud induction at same TDZ concentration regimes in both genotypes (p<0.02). The analysis of means revealed that the treatment 4.0 mg/l of TDZ was most effective for induction of multiple shoot buds per explants from both test explants and genotypes (Table 2).

Recently, Shekhar *et al.* [9] have titrated different TDZ concentration levels (1 to 6 mg/l) for shoot bud induction from mature zygotic embryo explants of pigeonpea. They identified 4.0 mg/l TDZ as most effective for multiple shoot induction from mature zygotic embryo explants in pigeonpea. Similarly, the results of present study also revealed that any further increase in TDZ concentration levels imparts reduction in multiple shoot bud induction in both tested explants of pigeonpea (Figs. 2 and 3). The same kind of explant response to TDZ levels in growth medium has been reported in cotyledonary node of *Cassia sophera* and mature zygotic embryo explants of pigeonpea [9, 10]. Further, TDZ (1.0 mg/l) in combination to 2,4-D (1 mg/l) have been found effective for shoot generation from cotyledon and hypocotyl explants of *L. campestre* as well [11]. Earlier studies indicated that embryonic axis with cotyledon are best for producing maximum of 55.02 shoots per explant when cultured on MSB5 medium with 2 mg/l 2-isopentenyladenine (2-iP), 1 mg/

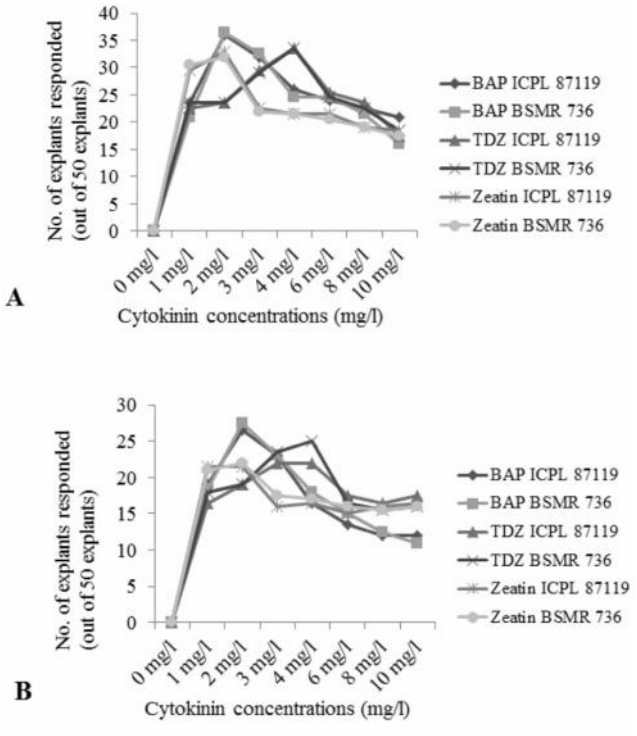

Fig. 2. Response pattern of explants cultured on medium augmented with different cytokinins for multiple shoot induction in ICPL 87119 and BSMR 736 pigeonpea genotypes. A. Number of cotyledonary node with cotyledons explants responded to increasing cytokinin concentration regimes. B. Number of embryo discs with half cotyledon explants responded to increasing cytokinin concentration regimes

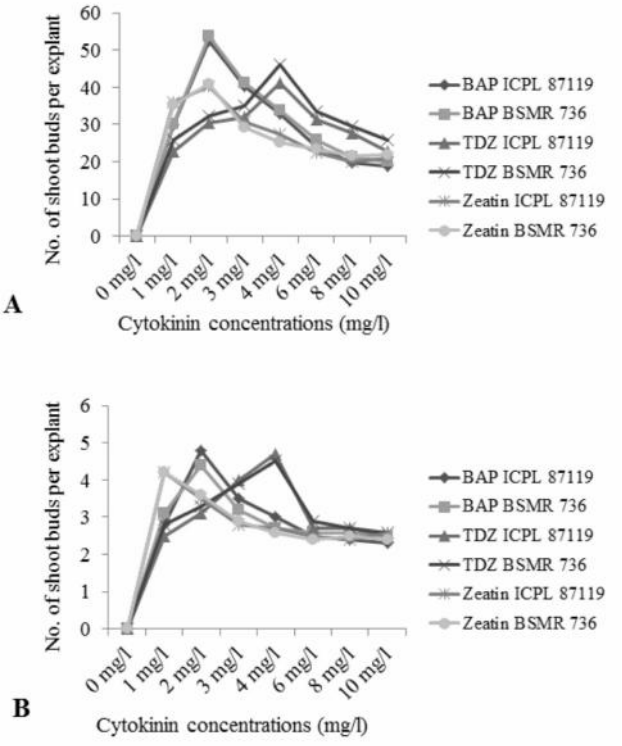

Fig. 3. Multiple shoot bud induced per explant cultured on medium supplemented with different cytokinins in ICPL 87119 and BSMR 736 pigeonpea genotypes. A. Number of shoot buds induced per cotyledonary node with cotyledons explant in response to increasing cytokinin concentration regimes. B. Number of shoot buds induced per embryo discs with half cotyledon explants in response to increasing cytokinin concentration regimes

Table 3. Effect of BAP, TDZ and zeatin on direct multiple shoot induction from embryo discs with half cotyledon explants of pigeonpea genotypes, ICPL 87119 and BSMR 736, after 12 days of culture (50 explants)

Growth hormone concentrations	Number of explants responded (mean ±SD)					
	BAP		TDZ		Zeatin	
	ICPL 87119	BSMR 736	ICPL 87119	BSMR 736	ICPL 87119	BSMR 736
0 mg/l	0.0±0.0^{e}	0.0±0.0^{e}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{d}
1 mg/l	19.0±1.4^{bc}	18.5±3.5^{bc}	16.5±2.1^{b}	18.0±2.8^{b}	21.0±3.5^{a}	21.0±1.4^{ab}
2 mg/l	26.5±2.1^{a}	27.5±2.1^{a}	19.0±1.4^{b}	19.0±1.4^{b}	21.5±4.9^{a}	22.0±2.8^{a}
3 mg/l	23.0±1.4^{ab}	23.0±2.8^{ab}	22.0±2.8^{a}	23.5±2.1^{a}	16.0±2.8^{b}	17.5±3.5^{abc}
4 mg/l	16.5±2.1^{cd}	18.0±1.4^{bc}	22.0±5.6^{a}	25.0±1.4^{a}	16.5±2.1^{b}	17.0±1.4^{bc}
6 mg/l	13.5±2.1^{d}	15.0±1.4^{cd}	17.5±3.5^{b}	16.5±2.1^{b}	15.0±4.1^{b}	16.0±1.4^{c}
8 mg/l	12.0±1.4^{d}	12.5±0.7^{d}	16.5±2.1^{b}	15.5±0.7^{b}	16.0±1.4^{b}	15.5±1.2^{c}
10 mg/l	12.0±2.8^{d}	11.0±1.4^{d}	17.5±0.7^{b}	16.0±2.4^{b}	16.5±2.1^{b}	16.0±1.4^{c}

l thidiazuron (TDZ) and 0.4 mg/l kinetin (KIN) in chickpea (*Cicer arietinum* L.) [12]. Although, in present study, the best TDZ concentration observed for more number of shoot induction seems to be high (4.0 mg/l) however, in previous study same TDZ concentration level has been reported for more number of shoot induction from mature zygotic embryo in pigeonpea [9]. Further we also did not observe any artefacts such as deformed shoots when explants were cultured on MS augmented with different TDZ concentration regimes.

Multiple shoot bud induction in response to zeatin concentration regimes

The augmentation of MS with different zeatin concentration regimes resulted in the multiple shoot bud induction in cotyledonary node with cotyledon explants, which ranged from 0 to 33.0 in ICPL 87119 and from 0 to 32.0 in BSMR 736 (Table 1). In both genotypes, the maximum number of explants responded for shoot bud induction was recorded at 2.0 mg/l zeatin concentration regime. Further, the maximum number of shoot bud induced per explant was recorded at 2.0 mg/l zeatin concentration level, which was 41.1 for ICPL 87119 and 46.1 for BSMR 736 (Table 2). The maximum number of explants responding was recorded in at 2.0 mg/l zeatin concentration regime, which was 21.5 in ICPL 87119 and 22.0 in BSMR 736 (Table 3). The number of shoot bud induced from embryo discs with half cotyledon explant was ranged from 0 to 4.2 in both genotypes (Table 2). From embryo discs with half cotyledon explants, at 1.0 mg/l zeatin concentration regime the highest number shoot buds was recorded. The effect of change of zeatin concentration on shoot bud induction was similar to that of effect of change in BAP or TDZ concentration levels (Figs. 2 and 3). The observation of development of white sugary callous might be the reason for reduction in multiple shoot buds, when medium is supplemented with higher concentrations of cytokinins (Fig. 1C). Similar zeatin levels (2.0 mg/l) in growth medium have been documented as most effective for shoot regeneration from cotyledonary node in pigeonpea [13]. The zeatin (2.0 mg/l) in combination to 2,4-D (1.0 mg/l) have been reported for shoot generation from cotyledon and hypocotyl explants of *L. campestre* [11].

The t-test statistical analysis indicated that the response of two test genotypes at same level of zeatin was non-significant ($p>0.84$). At the same time, there was a significant difference between the two explants response to same level of zeatin concentration regimes for multiple shoot bud induction ($p<0.03$). Similarly, it was observed that there was no significant difference between two test genotypes response for number of shoot bud induced per explants at same zeatin concentration regime ($p>0.96$). Whereas, the two explants (cotyledonary node with cotyledons and embryo discs with half cotyledon) noticed differ significantly for their response to shoot bud induction at same zeatin concentration regime in both genotypes ($p<0.001$). The analysis of means showed that the two treatments, 1.0 mg/l and 2.0 mg/l zeatin, were on par for number of explants responded to zeatin fortification (Tables 1 and 2). On other hand, the treatment with 2.0 mg/l zeatin level was superior over

other treatments in cotyledonary node with cotyledons explants and the treatment with 1.0 mg/l zeatin level was superior in embryo discs with half cotyledon explants for number of shoot bud induced per explant (Table 2).

Present study reports induction of more number of shoot buds in cotyledonary node with cotyledons explants when compared with that of embryo discs with half cotyledon explants (Fig. 3). The cotyledonary node with cotyledons explants has immense potential for multiple shoot bud induction. It is interesting to notice such difference, and might be it was due to removal of half cotyledon retaining embryo discs with other half part of it. Such detachment of half cotyledon might have resulted in reduction in cotyledonary node compactness, which might be essential for more number of shoot bud initiation. Although there are no previous reports indicating this phenomenon, based on present experimental results it seems obvious. Many individual studies have reported the response of different explants to multiple shoot bud induction in response to cytokinin fortification [7, 8]. As per our understanding, this is the first report indicating such huge number of multiple shoot bud induction from cotyledonary node with cotyledons explants in pigeonpea.

Rooting and establishment of plantlets

Elongated and well developed shoots (>3 cm long) were excised from shoot clumps to basal MS medium and cultured on MS medium augmented with increasing IBA concentration regimes. The frequency of rooting varied with different IBA concentration regimes ranging

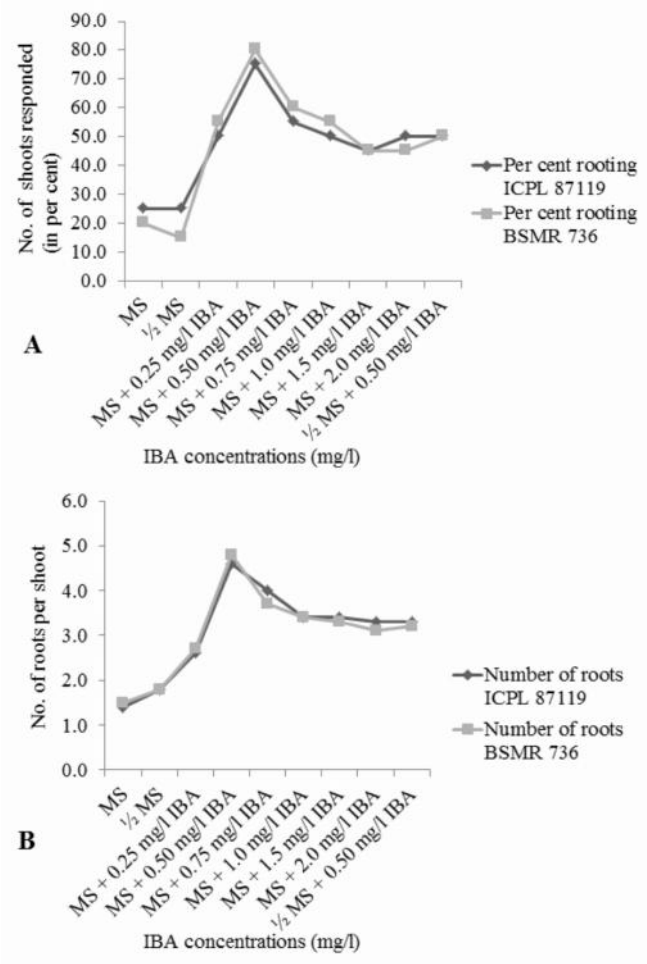

Fig. 4. Effect of MS nutrient media strength and different IBA concentration regimes on rooting A. The per cent shoots responded to rooting on MS medium fortified with different IBA concentration levels. B. Number of roots induced per shoot cultured on MS medium fortified with different IBA concentration levels

Table 4. Effect of IBA concentration regimes on root induction and number of root induced per shoot in pigeonpea genotypes, ICPL 87119 and BSMR 736, after 10 days of culture

Growth hormone concentrations	Per cent rooting (mean ± SD)		Number of roots per shoot (mean ± SD)	
	ICPL 87119	BSMR 736	ICPL 87119	BSMR 736
MS	25.0±7.0^{c}	20.0±8.0^{c}	1.4±0.5^{e}	1.5±0.4^{e}
½ MS	25.0±8.0^{c}	15.0±8.0^{c}	1.8±0.4^{e}	1.8±0.4^{e}
MS + 0.25 mg/l IBA	50.0±6.0^{b}	55.0±5.0^{b}	2.6±0.5^{d}	2.7±0.5^{d}
MS + 0.50 mg/l IBA	75.0±7.0^{a}	80.0±4.0^{a}	4.6±0.8^{a}	4.8±0.7^{a}
MS + 0.75 mg/l IBA	55.0±5.0^{ab}	60.0±6.0^{b}	4.0±0.7^{b}	3.7±0.5^{b}
MS + 1.0 mg/l IBA	50.0±7.0^{b}	55.0±5.0^{b}	3.4±0.6^{c}	3.4±0.4^{bc}
MS + 1.5 mg/l IBA	45.0±5.0^{bc}	45.0±7.0^{b}	3.4±0.7^{c}	3.3±0.7^{bc}
MS + 2.0 mg/l IBA	50.0±4.0^{b}	45.0±6.0^{b}	3.3±0.6^{c}	3.1±0.5^{cd}
½ MS + 0.50 mg/l IBA	50.0±6.0^{b}	50.0±7.0^{b}	3.3±0.7^{c}	3.2±0.4^{c}

from 20 to 80 % in both genotypes (Table 4). The highest root induction was noticed in MS media with 0.5 mg/l IBA. The observed number of roots per shoot ranged from 1.4 to 4.8 per shoot. The maximum number of roots induced per shoot was recorded in MS fortified with 0.5 mg/l IBA, in both genotypes. The induced roots were thick, white in color with less fine root hair developed on it (Fig. 1). The analysis of means revealed that the response to root induction and number of roots induced was maximum in MS supplemented with 0.5 mg/l IBA for both test genotypes (Table 4). Results of present study clearly indicated that the MS with 0.5 mg/l IBA was most suitable for healthy root induction with comparatively more number of roots (Fig. 4). Further increase in IBA levels in rotting medium did not affect the root induction significantly. Many studies have documented the effectiveness of lower concentrations of IBA on root induction in pigeonpea [7, 14].

Pigeonpea is among the most important grain legumes, ranks sixth in production. This crop suffers from many biotic and abiotic stresses, and the level of resistance in world germplasm accessions is low to moderate. Hence, there is a great need and opportunity to use tissue culture base methods to improve pigeonpea crop resistance to different stresses. Present study reports the efficient multiple shoot bud induction and plantlet regeneration method for pigeonpea. As pigeonpea genotypes used in present study, ICPL 87119 and BSMR 736, are moderate to resistant to *Fusarium* wilt and sterility mosaic disease, *in vitro* plantlet regeneration methods in such genotypes can be exploded for genetic engineering programs to develop multiple stress tolerance in pigeonpea.

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References

1. **Snapp S. S., Rohrbach D. D. and Simtowe F.** 2002. Sustainable soil- management options for Malawi: can smallholder farmers grow more legumes? Agri. Ecosys. Environ., **91**: 159-174.
2. **Singh U. and Eggum B. O.** 1984. Factors affecting the protein quality of pigeonpea (*Cajanus cajan* (L.) Millsp.), Qual Plantarum/Plant Food for Human Nutrition, **34**:251-261.
3. **Parde V. D., Sharma H. C. and Kachole M. S.** 2012. Protease inhibitors in wild relatives of pigeonpea against the cotton bollworm/legume pod borer, *Helicoverpa armigera*, American J. of Plant Sci., **3**:627-635.
4. **Kumar A. S., Reddy T. P. and Reddy G. M.** 1983. Plantlet regeneration from different callus cultures of pigeon pea (*Cajanus cajan* L.). Plant Sci. Lett., **32**: 271-278.
5. **Franklin G., Jeyachandran R., Melchias G. and Ignacimuthu S.** 1998. Multiple shoot induction and regeneration of pigeonpea (*Cajanus cajan* (L) Millsp.) cv. Vamban 1 from apical and axillary meristem. Current Sci., **74**: 936-937.
6. **Srinivasan T., Verma V. K. and Kirti P. B.** 2004. Efficient shoot regeneration in pigeonpea [*Cajanus cajan* (L) Millisp.] using seedling petioles. Current Sci., **86**: 30-32.
7. **Geetha N., Venkatachalam P., Prakash V. and Lakshmisita G.** 1998. High frequency induction of multiple shoots and plant regeneration from seedling explants of pigeonpea (*Cajanus cajan* L.). Current Sci., **17**: 1036-1041.
8. **Kashyap V., Saranji B. K., Yadav M. K. and Yadav D.** 2011. Assessment of *in vitro* multiple shoot bud induction from leaf explants among eleven Indian cultivars of pigeon pea (*Cajanus cajan* L. Milli sp.). Biotechnol., **10**(6): 534-539.
9. **Shekhar G. P. V., Venkateshwarlu M., Srilatha T., Reddy K. J. and Ugandhar T.** 2012. Plantlet regeneration from mature zygotic embryo culture of Pigeon pea (*Cajanus cajan* l. Mill sp) using thidiazuron. Inte. J. Pharma Bio. Sci., **3**(2): 300-305.
10. **Parveen S. and Shahzad A.** 2010. TDZ–induced high frequency shoot regeneration in *Cassia sophera* Linn. *via* cotyledonary node explants. Physiol. Mol. Biol. Plants, **16**(2): 201-206.
11. **Ivarson E., Ahlman A., Li X. and Zhu L.** 2013. Development of an efficient regeneration and transformation method for the new potential oilseed crop *Lepidium campestre*. BMC Plant Biol., **13**: 115.
12. **Pawar B. D., Jadhav A. S., Kale A. A., Chimote V. P. and Pawar S. V.** 2012. Thidiazuron improves *in vitro* multiple shoot induction in chickpea (*Cicer arietinum* L.) cotyledon with embryonic axis. J. Food Legumes **25**(1): 9-13.
13. **Srivastava J., Raghav P. K. and Rishi A.** 2013. Effect of different cytokinins on shoot induction in Pigeonpea (*Cajanus cajan* l.) Manak (h77216) variety. Int. J. Pharm Bio Sci., **4**(3): 55-63.
14. **Guru Prasad M., Prasad T. N. V. K. V. and Sudhakar P.** 2011. *In vitro* proliferation of shoot regeneration from embryo of *Cajanus cajan* L (var.LGG-29). J. Devel. Biol. Tissue Eng., **3**(5): 62-65.