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ESTABLISHMENT OF EFFICIENT *IN VITRO* CULTURE AND PLANTLET REGENERATION IN TWO SPECIES OF VIGNA: *VIGNA MUNGO* AND *VIGNA RADIATA*

Potshangbam Nongdam[1], Heisnam Rameshwari Devi[2]

[1,2]*Department of Biotechnology, Manipur University, Canchipur, Imphal, India*

ABSTRACT

The present study was conducted for the establishment of efficient in vitro regeneration protocols for Vigna mungo and Vigna radiata for large scale propagation. Different growth regulator combinations and concentrations were tested to study the shooting and rooting response and subsequent plantlet development. Presence of thidiazuron (TDZ) (1mg/L) and activated (AC) (0.5%) influenced the shooting and rooting initiation in both the legumes. The best shoot generation for Vigna mungo was observed in MS+3mg/L BAP+1mg/L TDZ+AC(0.5%) while the most prolific root development was recorded in MS+1.5mg/L BAP+3mg/L 2,4-D+1mg/L NAA+1mg/L TDZ+AC(0.5%). TDZ promoted shooting development though there was observation of negative influence on rooting in black gram. Whereas the maximum shoot multiplication in Vigna radiata was found in MS+2.5mg/L BAP+2.5mg/L NAA+AC(0.5%), the best rooting response was evident in MS+1.5mg/L BAP+3.5mg/L IBA+AC. TDZ exhibited unfavourable response to in vitro shooting and rooting response in Vigna radiata.

Keywords: activated charcoal, thidiazuron, callus, legumes, hardening

I. INTRODUCTION

Legumes are one of the most important and first cultivated crop plants. Their benefits are recognised around the world as human food, animal feed, for extracting vegetable oils, improving the soil nitrogen content etc. They are economical source of proteins for humans that add variety to the diets [1]. The legume seeds are generally characterized by relatively large content of protein (17 to 40%) and even greater concentration of carbohydrates and small amount of oil. *Vigna mungo* or Black grams and *Vigna radiata* or Green grams are highly valued plants for human and animal food. They are most imperative food legumes grown and consumed by humans [2]. Black gram is a tropical leguminous plant which belongs to genus *Vigna* and under sub genus Ceratotropis. It is highly prized pulse, very rich in phosphoric acid and is one of the most nutritious of all pulses. Black gram has protein content almost three times that of cereals and is one of the richest sources of proteins and Vitamin B [3]. It also has good quantities of iron, folic acid, calcium, magnesium, potassium. The rich fibre content also makes them easy to digest and helps to reduce cholesterol and improves cardiovascular health. The *Vigna radiata* or

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Green gram also forms a very nutritious article of diet and is consumed in the form of whole dried seeds and dal prepared by splitting the seeds in a mill [4]. The sprouted beans are highly nutritious food and are one of the most wholesome among pulses in India. Both *Vigna mungo* and *Vigna radiata* are among the most important legumes used as vegetables, pulses and feed for human and animal in developing countries. Because of their great value, there is a huge demand for large scale production of these legumes. But the conventional methods of crop propagation may not suffice the present requirement due to ever increasing population pressure and alarming decreased in available land resources. Also there are limitations in the crop production due to various biotic pressures. The high susceptibility of these legumes to yellow mosaic virus (YMV), fungal pathogens, insects and drought result in significant yield losses [2]. The *in vitro* regeneration of these legumes through tissue culture provides an excellent opportunity for propagating them at large scale. However legumes in general are recalcitrant to tissue culture and are highly genotype specific [5], so regeneration through *in vitro* culture techniques has been quite difficult for these plants. There are very few reports on efficient regeneration system of these two species which is prerequisite for large scale propagation. The present study was conducted with an aim to establish efficient, fast and reliable *in vitro* regeneration protocols for mass propagation of *Vigna mungo* and *Vigna radiata*.

II. MATERIALS AND METHODS

The experimental materials (seeds) of *Vigna mungo* and *Vigna radiata* were collected from Central Agriculture University (CAU), Iroishemba, Manipur for the establishment of culture. The seeds were first washed thoroughly in running tape water with 20% teepol as wetting agent for 30-45 minutes followed by treatment with 70% ethanol for 1 minute. The treated seeds were washed 3-4 times with double sterile distilled water before being subjected to 0.2% mercuric chloride treatment for 4-5 minutes. The seeds were finally rinsed 5-6 times with sterile distilled water to remove any mercuric chloride present on the surface. The surface sterilization of seed explants was conducted in aseptic condition under laminar hood cabinet and the sterilised seeds were used for *in vitro* culture initiation. The Murashige and Skoog medium [6] was employed for *in vitro* culture and plantlet generation of *Vigna* species. Separate stock solutions were prepared for macro and micro-nutrients, vitamins, hormones and iron salts in a concentrated solution and stored at 25-30ºC until use. Culture medium was prepared by adding the macro and micro nutrients, vitamins and other salts from the stock solutions one after another into a conical flask of desired size followed by volume make up with sterile distilled water as per the requirement. The pH was adjusted at 5.8 after adding 2% sucrose and the medium was boiled with 0.9% agar as gelling agent. Activated charcoal (0.5%) was incorporated into the medium which required sufficient swirling of culture vessels to completely dissolve it. Definite quantities of medium were dispensed to culture tubes after growth regulators were added and culture vessels were tightly closed with appropriately sized cotton plugs. The medium were autoclaved finally at 121ºC for 15 minutes and placed in an appropriate position either vertical or slanting to allow sufficient medium gelling. The medium was supplemented with different concentrations of indole-3-butyric acid (IBA), 1-nathyl acetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-benzyl amino purine (BAP)) in varied combinations to study *in vitro* shooting and rooting responses leading to plantlet development. Influence of Thidiazuron (TDZ) (1mg/L) on shoot and root development was

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also studied by incorporating it in different growth hormone combinations tested. The cultures were maintained inside culture room in controlled condition with proper light illumination of 60 $\mu molm^{-2}$ s^{-1} for 12 hours a day using white fluorescent tubes and temperature at 25±2°C.

The well rooted plantlets after 4 weeks of culture were rinsed with water to wash off the agar medium and transferred to small pots containing a mixture of soil, sand and manure (1:1:1). The hardened plants were maintained at 24±2°C with 18 h photoperiod before they were transferred to green house for further hardening. The plants developed an efficient root system during this period with full blown photosynthetically active leaves enabling them to develop terrestrial water control. Healthy plantlets of 7-10 cm in height were planted in bigger pot with mixture of garden soil, sand and cowdung at the ratio of 2:1:1.

The experiment was conducted twice with four replicates per hormonal combinations. The observation was made at regular intervals after every week and number of leaf and root development in each treatment was recorded. The data obtained from the present investigation were subjected to Analysis of Variance (ANOVA) and significant differences were determined by employing Duncan's multiple range test at p=0.05 [7]. The statistical data analysis was performed using SPSS software program (SPSS Inc., Chicago, USA).

III. RESULTS

MS medium supplemented with different plant growth hormones viz., auxin and cytokinins were employed for *in vitro* regeneration of the two selected legumes i.e., *Vigna mungo* and *Vigna radiata.* Influence on the *in vitro* shooting and rooting response was also studied in the two legumes by incorporating AC (0.5%) and TDZ (1mg/L) in different hormonal combinations tested.

3.1. SHOOT AND ROOT INDUCTION IN *VIGNA MUNGO*

The different growth regulator combinations and concentrations strongly influenced the germination and organogenic potential of the seed explants. Efficient shoot regeneration has been achieved on MS medium supplemented with 1mg/L TDZ. Presence of BAP with other growth regulators enhanced shoot induction and multiplication. Supplementation of 3mg/L BAP with 1mg/L TDZ produced rapid shoot multiplication and maximum shoot number in the culture (Fig.1a & TABLE-1). Incorporation of IBA at higher concentration hampered shoot initiation response and lower shoot formation was observed in MS+2mg/L BAP+3mg/L IBA+1mg/L TDZ (TABLE-1). Supplementation of 0.5% AC in all the growth regulators combinations tested showed reduced shoot formation with maximum reduction found in MS+3mg/L BAP+1mg/L TDZ (from 4.2±1.4 to 2.1±0.8) (Fig.2). This response indicated the negative influence of AC on shoot induction when TDZ was present in the combination. Least shoot number formation was observed in MS + 2mg/L BAP + 3mg/L IBA + 1mg/L TDZ + 0.5% AC (TABLE-2).

The rooting response was significantly affected by the auxin concentration, presence of TDZ and AC in the combinations. Lower rooting initiation was recorded in all the growth hormone combinations with TDZ(1mg/L) except for basal MS medium and MS+1.5mg/L BAP + 3.5mg/L 2,4-D+1mg/L NAA which produced 7.2±1.5 and 14.7±1.5 number of roots respectively (TABLE 1).Incorporation of AC (5%) showed drastic increase in

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rooting response as evidenced from high rooting proliferation in MS+2.5mg/L BAP+2.5mg/L NAA+1mg/L TDZ (from 0 to 6.7±1.7), MS +3.5mg/L 2,4-D+1mg/L TDZ (from 0 to 9.5±2.0), MS+2mg/L BAP+3mg/L IBA +1mg/LTDZ (from 4.5±1.2 to 10.2±2.5) and MS+1.5mg/L BAP + 3.5 mg/L 2,4-D+1mg/L NAA+1mg/L TDZ (from 14.7±1.5 to 19.2±2.2) (Fig.1b & Fig.3).

3.2. CALLUS INDUCTION IN *VIGNA MUNGO*

The combination of BAP, 2,4-D and NAA or their presence individually exhibited prominent influence on mode of callus induction. Significant callus formation was observed in medium supplemented with 3.5mg/L 2,4-D (Fig.1c). AC incorporated medium showed decreased callus production as observed in MS+3.5mg/L 2,4-D+AC. Both the auxins (2,4-D and NAA) at higher concentration with BAP induced callusing at the lower ends of seed explants. The nature of callus differed with the type of auxin as 2,4-D induced yellow creamish friable callus whereas NAA induced light brown calli.

3.3. SHOOT AND ROOT GENERATION IN *VIGNA RADIATA*

Shooting initiation in response to different growth regulator combinations was studied at different concentrations. AC (0.5%) was incorporated in all the growth regulator combinations tested. Shooting was enhanced with increased concentration of BAP as shoot number significantly improved when BAP concentration increased from 1mg/L to 2.5mg/L in presence of 2.5mg/L NAA and AC (TABLE-3). Multiple shoot development and the best *in vitro* shooting response were observed in MS+2.5mg/L BAP+2.5mg/L NAA+AC (Fig.1d). Supplementation of 1mg/L TDZ in the hormonal combinations investigated showed less effectiveness in promoting shooting in culture. There was decline in shoot regeneration with TDZ with reduction evidenced in MS+2.5mg/L BAP+2.5mg/L NAA+1mg/L TDZ+AC (from 4.1±1.0 to 2.1±0.2) and MS+1.5mg/L BAP+3.5mg/L IBA+AC (from 3.0±0.7 to 2.1±0.6) (Fig.4). The influence of TDZ on shooting response was marginal and not significant in culture grown in MS+1mg/L BAP+2.5mg/L NAA+1mg/L TDZ+AC and MS+2.5mg/L BAP+1.5mg/L IBA+1mg/L TDZ+AC as shoot number generated remained more or less the same with or without TDZ (TABLE-4).

In vitro rooting response was influenced by presence of higher concentration of IBA and NAA along with AC. Root multiplication was prominent in MS+1.5mg/L BAP+3.5mg/L IBA+AC and maximum root number was recorded in the combination (Fig.1e). Auxin concentration lower than or equal to cytokinin (BAP) in the medium could not significantly influence higher rooting multiplication. The root generation in MS+2.5mg/L BAP+1.5mg/L+AC and 2.5mg/L BAP+2.5mg/L NAA+AC was significantly lower as compared to root formation observed in MS+1.5mg/L BAP+3.5mg/L IBA+AC and MS+1.5mg/L BAP+3mg/L 2,4-D+AC (TABLE-3). Incorporation of TDZ (1mg/L) in some hormonal combinations produced drastic reduction in the rooting response as observed in MS+1.5mg/L BAP+3.5mg/L IBA+AC (from 10.7±2.2 to 3.2±0.8) and MS+1mg/L BAP+2.5mg/L NAA+AC (from 4.2±0.7 to 1.5±0.2) (Fig.5). This indicated the negative influence of TDZ in rooting even when growth regulators favourable for rooting were also present. The seedlings with healthy leaves and roots were removed from the culture tubes and subsequently hardened for transplantation to greenhouse conditions (Fig.1f).

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IV. DISCUSSIONS

Various studies have been carried out on *in vitro* plantlet regeneration of *Vigna mungo* and *Vigna radiata* and different results of growth in response to culture conditions have been observed. Geetha *et al*. [8] reported maximum callus development with shoot tip explants while hypocotyl produced best callusing [9]. The present study showed that callus induction was found to be appropriate for seed explants in 3.5mg/L 2,4-D supplemented medium. Mathur and Prakash [10] also reported significant callus induction in *Vigna mungo* on MS medium supplemented with 2.0 mg/L 2,4-D and 0.5 mg/L Kinetin. But in our observation 2,4-D alone at higher concentration could give the same callusing response. The shooting frequency in *Vigna mungo* was 83.3% when 4.0mg/L BAP was singly employed [11]. But in the present investigation BAP in combination with NAA and TDZ produced similar response. Mony *et al.* [12] reported the lowering of BAP concentration (1.0mg/L) produced maximum number of shoots in black gram but different response was observed in present study with higher shoot formation in increase concentration of BAP at 2.5mg/L and 3.0mg/L. The different response might be due to presence of TDZ and other additive like AC. The maximum shooting response in *Vigna mungo* was observed on medium supplemented with highest cytokinin and lower auxin concentration. But in *Vigna radiata,* the best shooting response was received in higher cytokinin and auxin concentration. Lowering of concentration of BAP produced reduce shoot formation in the culture. The frequency of shooting thus seemed to depend on concentration of BAP and the same finding was also reported by Khera and Mathias [13]. TDZ enhanced shooting in *Vigna mungo* though its presence in the medium was inappropriate for shoot induction in *Vigna radiata*. Chandra and Pal [14] reported that MS medium containing 0.25mg/L IBA performed best for rooting in four weeks in *Vigna* species. Geetha *et al*. [15] also observed a higher percentage of rooting (100%) with 0.5mg/L IBA. Raman *et al*. [9] reported efficient rooting (100%) of shoots on medium containing half MS salts, full MS vitamins and IBA (0.5mg/ L). High rooting (78.3%) was also observed in MS medium containing 3.0 mg/L IBA [15]. In our study, IBA incorporated medium showed the second best rooting response in *Vigna radiata* while maximum rooting was observed in NAA and 2,4-D enriched medium with lower concentration of BAP. In *Vigna mungo* higher rooting response was evident in medium supplemented with 2, 4-D and BAP. The presence of TDZ produced significant decline in root induction in both the legumes inspite of presence of favourable root initiating growth hormones. This may be due to the antagonistic nature of TDZ to root promoting hormones producing detrimental effects to culture growth and root development.

V. CONCLUSION

The *in vitro* propagation of *Vigna mungo* and *Vigna radiata* carried during the present study showed enhanced shoot formation with BAP while rooting response was better in IBA and NAA enriched medium. The well rooted seedlings of both the *Vigna* species were subjected to proper hardening by thoroughly washing with water and transferring them to small community pots containing soil, sand and cow dung in proper proportion. The present investigation successfully established the *in vitro* protocols for effective shooting and rooting development leading to complete plantlet generation from seed explants of *Vigna mungo* and *Vigna radiata*. The *in vitro* regeneration protocols presently developed will help in the generation of large number of quality materials for these two legumes. This kind of efficient *in vitro* regeneration systems would also be useful for

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development of improved transgenic black and green grams via micro projectile bombardment or *Agrobacterium* – mediated gene transformation as they are of high economical source of proteins for human diets.

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TABLE 1: *In vitro* shoot, root and leaf formation of *Vigna mungo* cultured on MS medium supplemented with different growth hormone combinations and concentrations

Plant Growth Hormone combinations	No. of shoots	No. of roots	No. of leaves
MS+1mg/L TDZ	2.0±1.0^{e}	7.2±1.5^{e}	4.0±1.0^{c}
MS + 3 mg/ L BAP+1mg/L TDZ	4.2±1.4^{d}	2.1±0.7^{d}	3.6±2.5^{d}
MS + 2.5 mg/L BAP +1mg/ L IBA+1mg/ L TDZ	4.1±1.0^{a}	2.5±1.1^{c}	3.1±1.7^{a}
MS + 2 mg/ L BAP + 3mg/ L IBA+1mg/L TDZ	1.6±0.5^{c}	4.5±1.2^{b}	4.2±2.5^{c}
MS+2.5m/gL BAP + 2.5mg/L NAA+1mg/L TDZ	3.1±1.1^{a}	-	4.1±1.1^{c}
MS+3.5mg/L 2,4-D + 1mg/L TDZ	1.2±0.6^{b}	-	1.5±0.6^{b}
MS+1.5 mg/L BAP + 3.5mg/ L 2,4-D +1mg/L NAA+1mg/L TDZ	3.0±1.0 ^{a}	14.7±1.5^{a}	3.1±1.7^{a}

The results are based on 4 replicates per treatment in two repeated experiments. Means followed by same letter are not significantly different at p =0.05, Hyphen (-) indicates the absence of *in vitro* root formation.

TABLE 2: *In vitro* shoot, root and leaf formation of *Vigna mungo* cultured on activated charcoal (0.5%) supplemented MS medium with different growth hormone combinations and concentrations

Plant Growth Hormone combinations	No. of shoots	No. of roots	No. of leaves
MS+1mg/ L TDZ +AC	1.8±1.1^{e}	-	3.5±0.8^{f}
MS + 3 mg/ L BAP+1mg/L TDZ+AC	2.1±0.8^{e}	1.5±0.5^{e}	4.0±1.4^{b}
MS + 2.5 mg/L BAP +1 mg/ L IBA+1mg/ L TDZ+AC	3.2±1.2^{d}	8.2±2.1^{d}	2.5±0.7^{e}
MS + 2 mg/ L BAP + 3mg/ L IBA+1mg/ L TDZ +AC	1.2±0.6^{c}	10.2±2.5^{b}	2.0±1.5^{d}
MS+2.5mg/L BAP + 2.5mg/L NAA+1mg/L TDZ+AC	2.2±0.8^{a}	6.7±1.7^{c}	1.5±0.2^{c}
MS+3.5mg/L 2,4-D + 1mg/L TDZ+AC	2.5±0.9^{b}	9.5±2.0^{b}	4.1±0.6^{b}
MS+1.5 mg/L BAP + 3.5mg/ L 2,4-D +1mg/ L NAA+1mg/ L TDZ+AC	2.2±1.0 ^{a}	19.2±2.2^{a}	4.3±1.7^{a}

The results are based on 4 replicates per treatment in two repeated experiments. Means followed by same letter are not significantly different at p =0.05, Hyphen (-) indicates the absence of *in vitro* root formation.

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TABLE 3: *In vitro* shoot, root and leaf formation of *Vigna radiata* on activated charcoal (0.5%) supplemented MS medium with different growth hormone combinations and concentrations.

Plant Growth Hormone combinations	No. of shoots	No. of roots	No. of leaves
MS+ 1mg/L BAP + 2.5mg/L NAA + AC	1.6±0.6^{e}	4.2±0.7^{e}	2.0±1.0^{c}
MS+ 2.5mg/L BAP + 2.5mg/LNAA+ AC	4.1±1.0^{d}	2.2±0.7^{d}	4.1±1.4^{a}
MS+1.5mg/L BAP + 3mg/L 2,4-D + AC	2.3±0.8^{c}	5.1±1.1^{c}	4.1±0.4^{a}
MS+1.5mg/L BAP + 3.5mg/L IBA + AC	3.0±0.7^{b}	10.7±2.2^{b}	4.3±0.6^{b}
MS+2.5mg/L BAP+ 1.5mg/ L IBA+ AC	3.5±0.9^{a}	3.5±0.6^{a}	4.2±1.6^{a}

The results are based on 4 replicates per treatment in two repeated experiments. Means followed by same letter are not significantly different at p =0.05

TABLE 4: *In vitro* shoot, root and leaf formation of *Vigna radiata* on activated charcoal (0.5%) and TDZ supplemented MS medium with different growth hormone combinations and concentrations.

Plant Growth Hormone combinations	No. of shoots	No. of roots	No. of leaves
MS+1mg/L BAP + 2.5mg/ L NAA+ 1mg/L TDZ + AC	1.6±0.2^{d}	1.5±0.2^{d}	-
MS+2.5mg/L BAP+ 2.5mg/L NAA+1mg/L TDZ + AC	2.1±0.2^{b}	5.0±1.2^{c}	4.1±1.4^{a}
MS+1.5mg/L BAP + 3mg/ L 2,4-D +1mg/L TDZ + AC	1.4±0.5^{c}	2.5±0.7^{b}	4.2±1.3b
MS+1.5mg/L BAP + 3.5mg/L IBA + 1mg/L TDZ + AC	2.1±0.6^{b}	3.2±0.8^{a}	4.1±1.1^{a}
MS+ 2.5mg/L BAP+1.5mg/L IBA+ 1mg/L TDZ + AC	3.5±1.6^{a}	-	4.1±1.2^{a}

The results are based on 4 replicates per treatment in two repeated experiments. Means followed by same letter are not significantly different at p =0.05; Hyphen (-) indicates the absence of *in vitro* leaf and root formation

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Figure 1(a-f): *In vitro* culture of *Vigna mungo* and *Vigna radiata*: (a) shoot multiplication of *Vigna mungo* in MS+3mg/L BAP+1mg/L TDZ;(b) root proliferation of *Vigna mungo* in MS+1.5mg/L BAP+3.5mg/L 2,4-D+1mg/L TDZ+ 0.5%AC; (c) *in vitro* callus induction in *Vigna mungo* on MS medium supplemented with 3.5mg/L 2,4-D; (d) *in vitro* multiple shoot generation of *Vigna radiata* in MS+2.5mg/L BAP+2.5mg/L NAA+0.5% AC; (e) rapid root development of *Vigna radiata* in MS+1.5mg/L BAP+3.5mg/L IBA+0.5% AC; (f) Hardened plantlets.

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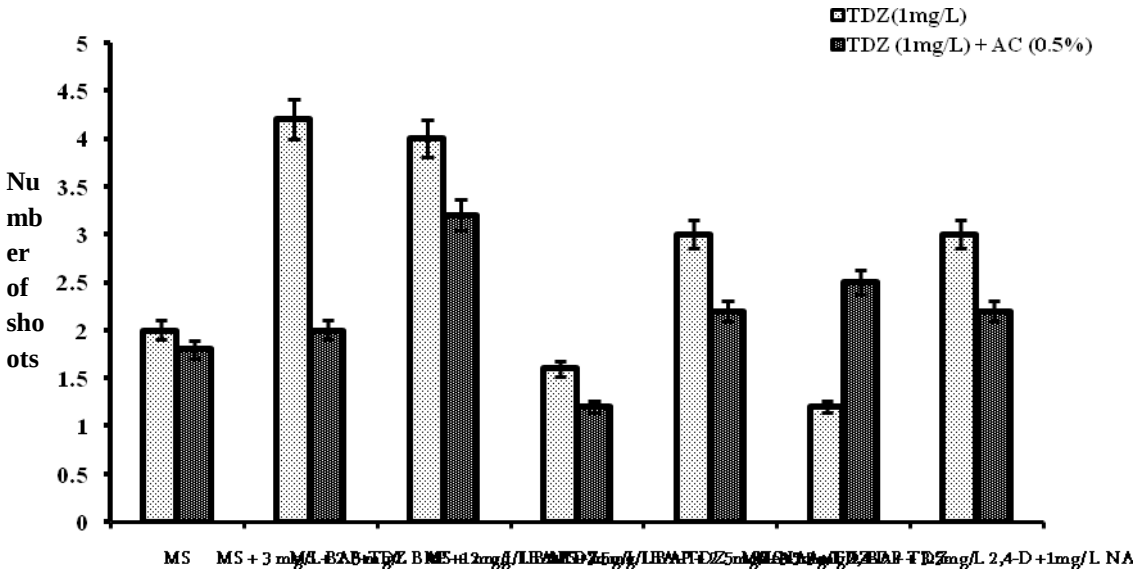

Figure 2: Influences of TDZ (1mg/L) and AC (0.5%) in the shoot formation of *Vigna mungo* on medium supplemented with different combinations and concentrations of plant growth regulators

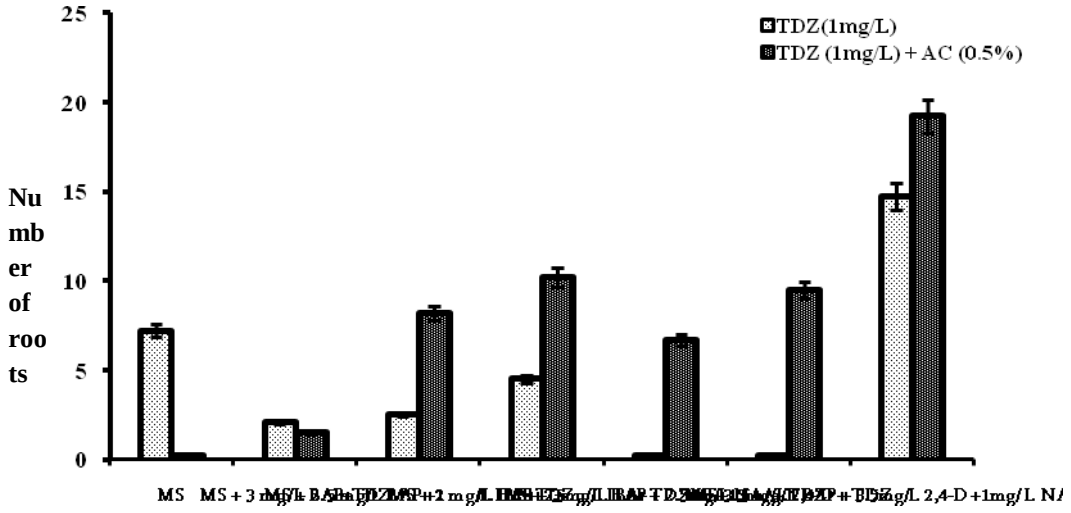

Figure 3: Influences of TDZ (1mg/L) and AC (0.5%) in the root formation of *Vigna mungo* on medium supplemented with different combinations and concentrations of plant growth regulators.

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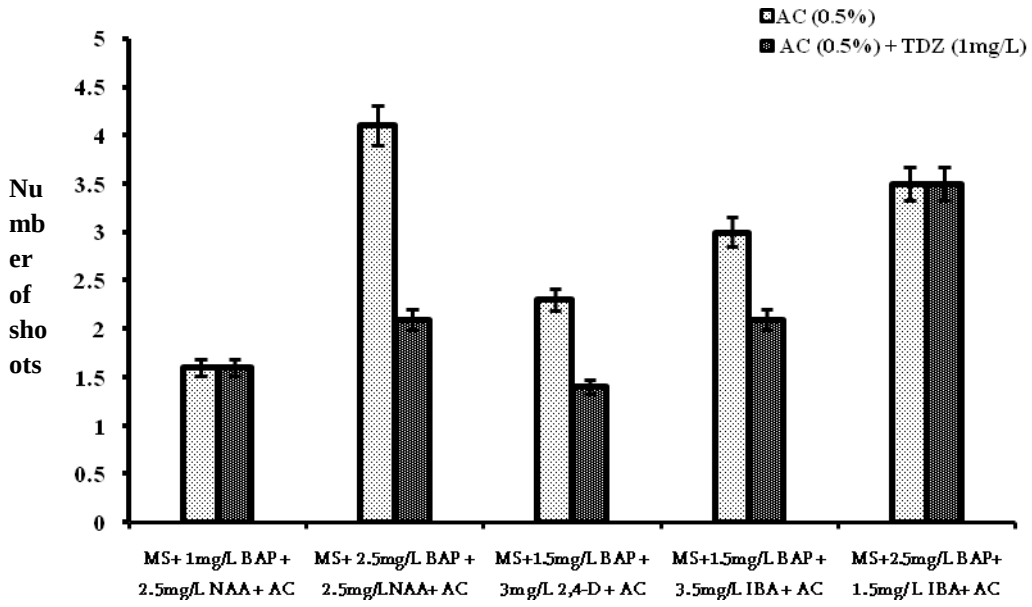

Figure 4: Influences of AC (0.5%) and TDZ (1mg/L) in the shoot formation of *Vigna radiata* on medium supplemented with different combinations and concentrations of plant growth regulators.

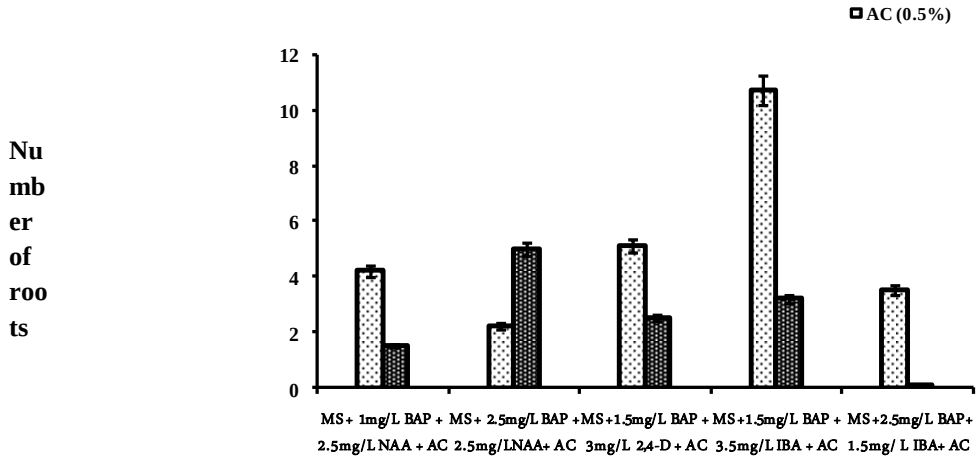

Figure 5: Influences of AC (0.5%) and TDZ (1mg/L) in the root formation of *Vigna radiata* on medium supplemented with different combinations and concentrations of plant growth regulators.