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Plant regeneration from mature cotyledon, embryo and hypocotyl explants of *Withania somnifera* (L.) Dunal.

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The explants with higher regeneration potential, mature cotyledon, embryo and hypocotyls explants of two genotypes of *Withania somnifera namely*: JA-20 and MWS-100 were cultured on MS basal media fortified with different concentrations and combinations of various auxins and cytokinins. Considering higher *in vitro* response, culture medium MS2D.5B (MS + 2.0 $mg.l^{-1}$ 2,4-D + 0.5 $mg.l^{-1}$ BA + 30.0 $g.l^{-1}$ sucrose + 7.5 $g.l^{-1}$ agar) initiated calli in higher frequencies from cultured mature cotyledon and embryo explants, while the hypocotyl explant, induction medium MS2N.5Kn (MS + 2.0 $mg.l^{-1}$ NAA + 0.5 $mg.l^{-1}$ Kn + 30.0 $g.l^{-1}$ sucrose + 7.5 $g.l^{-1}$ agar) was proved superior for callus initiation. For formation of morphogenic calli and plantlet regeneration nutrient medium MS2B.5N (MS + 2.0 $mg.l^{-1}$ BA + 0.5 $mg.l^{-1}$ NAA + 30.0 $g.l^{-1}$ sucrose + 7.5 $g.l^{-1}$ agar) were investigated remarkably superior. Higher *in vitro* rooting response was achieved on rooting medium (MS + 2.0 $mg.l^{-1}$ IBA + 15.0 $g.l^{-1}$ sucrose + 7.5 $g.l^{-1}$ agar). Higher *in vitro* morphogenic response was exhibited by explant mature embryo followed by explants mature cotyledon and hypocotyl. In terms of *in vitro* genotypic response genotype JA-20 was found significantly superior to MWS-100 for the most of the attributes investigated. Regenerated plantlets were established successfully in the field after hardening.

Keywords*:* *Withania somnifera*, plantlet regeneration.

Introduction

Withania somnifera (L.) Dunal, commonly known as Indian ginseng belongs to the family Solanaceae, having enormous medicinal and aromatic properties and has been included in ancient text of Ayurveda (Sharma *et al*., 2010). It is used as an abortifacient, amoebocide, anodyne, bactericide, contraceptive, diuretic and spasmolytic (Kurup, 1956; Asthana and Raina, 1989). Biological assays label the plant as having the properties against

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different diseases like leprosy, nervous disorders, diseases of respiratory and reproductory tract, venereal disorders, rheumatism, inflammation, psoriosis, bronchitis, asthma, consumption, ulcers, scabies, marasmus of children, insomnia, senile debility, alexipharmic, carbuncles, cancer, epilepsy, diabetes etc. (Tripathy *et al*., 1996; Kirtikar and Basu, 2001). Multiple uses of the plant have necessitated its large-scale compilation as raw material to the medicine industry, leading to over exploitation and making it an endangered plant species (Antonisame and Manikam, 1999). *Withania somnifera* (L.) Dunal can be propagated both by sexual and asexual method. Seed propagation, however is not always satisfactory, since the heterogenetically strain produces a great deal of variation. Again multiplication through cuttings give rise to less ramified plants and is consequently less productive than plants obtained from seeds (Supe *et al*., 2006). Tissue culture techniques can play an important role in the propagation and qualitative improvement of this medicinally important plant. In *Withania* like wise other crop species, efficient and frequent plant regeneration through embryogenic callus cultures is essential for novel crop improvement techniques based on somaclonal variation, *in vitro* selection, protoplast fusion and genetic transformation. During the last years, an array of efforts has been made to establish regenerable culture system in *Withania*. For this purpose, diverse explants have been employed to produce regenerable cultures *via in vitro* morphogenesis such as cotyledon (Rani *et al*., 2003), embryo (Anjali *et al*., 2000), hypocotyl (Rani and Grover, 1999; Anjali *et al*., 2000; Rani *et al*., 2003), shoot tips (Sen and Sharma, 1991 ; Supe *et al*., 2006), leaf disc (Rani *et al*., 2003; Sharma *et al*., 2010), root (Rani *et al*., 2003), apical bud (Sivanesan, 2007), nodal segments (Anjali *et al*., 2000; Sivanesan and Murugesan, 2008), auxiliary bud (Saritha and Naidu, 2007) with varying degree of success. Regarding the aforementioned, the objectives of present research were to develop a system of an efficient *in vitro* regeneration of Jawahar Asgandh -20 (JA-20) and Mandsaur *Withania somnifera* - 100 (MWS-100) varieties of commercial importance of Malwa-Plateu Zones of India, by means of the culture of diverse explants such as mature cotyledon and embryo and hypocotyl and compute optimum cytokinin-to-auxin ratio exhibiting *in vitro* morphogenesis in higher frequencies.

Materials and methods

Two genotypes of *Withania somnifera namely*: Jawahar Asgandh -20 (JA-20) and Mandsaur *Withania somnifera* - 100 (MWS-100) were chosen to conduct the present investigation. Both genotypes were obtained from All India Networking Project on Medicinal & Aromatic Crops, College of Horticulture,

Mandsaur, Rajmata Vijayaraje Scindia Krishi Vishwa Vidyalaya, Gwalior (M. P.).

To begin with a preliminary experiment, speckled fortifications of two basal media *viz*: MS (Murashige and Skoog, 1962) and B_5 (Gamborgs medium) (Gamborg 1968) were subjected to search out enhanced *in vitro* response. During the preliminary experimentations, MS basal medium (Murashige and Skoog medium) was found to be more responsive as compared to B_5 medium. Consequently MS was used as basal medium for main experimentations. Apart from MS basal micro and macro salts, vitamins, and agar powder, three auxins, namely: 2, 4-D (2,4-dichlorophenoxyacetic acid), NAA (α-Naphthalene acetic) and 2, 4, 5-T (2,4,5 trichlorophenoxyacetic acid) and three cytokinins *viz*: BAP(6-benzylaminopurine), Kn (kinetin) and TDZ (Thidizuron) in varying concentrations were added to fortify MS media for initiation of callus cultures from diverse explants. During the initial experiment, it was observed that an auxin as well as a cytokinin alone is not adequate for culture establishment leading to plantlet regeneration in higher frequencies. Furthermore, auxin 2, 4, 5-T responded scantily during initial experiments. As a result, for main experiment basal MS medium was fortified with varying concentrations and combinations of plant growth regulators (BAP, Kn and TDZ in combination with NAA and 2,4-D, 30g.l^{-1} sucrose and 7.5g.l^{-1} agar. Readymade MS basal medium, plant growth regulators and other ingredients were procured from Hi-Media® Laboratories, Mumbai, India.

Prior to explant excision, mature seed were washed with 2% Tween 20 (v/v) for 15-20 minutes and then washed thoroughly with running tap water for 10 minutes followed by a treatment with 70% (v/v) ethanol for 1 minute. Seeds were then subjected to treatment of 2% Bavistin® (a benzimidazole fungicide; BASF, Germany) followed by 0.2% $HgCl_2$ for five minutes each. Mature embryos explants were excised from pre-soaked seeds for 24 hours in sterilized double distilled water and mature cotyledon and hypocotyl explants from 4-7 days-old germinating seeds. Cultured 100x17mm glass Petri dishes with 7-8 pieces of explant were sealed with Parafilm® and incubated under complete darkness at 25±2°C for 1 week. Later the petridishes were subjected to 16 h photoperiod regime of 2000 lux luminance provided with cool white fluorescent lamps. After 4 -5 weeks of initial culturing, somatic embryoids and calli were sub cultured on same medium for regeneration of plantlets. When shoots of proper length was not attained they were subsequently transferred to MS elongation medium fortified with different concentrations of GA_3, 20g.l^{-1} sucrose and 7.5g.l^{-1} agar. Cultured baby food bottles /culture tubes were

subjected to 25±2°C temperature and photoperiod regimes of 60 μ mol m^{-2} s^{-1} luminance provided by cool fluorescent tubes for 16 hr. When rooting of regenerants was not occurred, plantlets were subsequently transferred to MS rooting medium supplemented with different concentrations of auxins (IBA, NAA and IAA), 15g.l^{-1} sucrose and 7.5 g. l^{-1} agar powder. For elongation and rooting, reduced level of sucrose was used on the basis of work conducted by various scientists as well as preliminary experience of this laboratory. Rooted plants were thoroughly washed with running tap water to remove the adhering agar and were planted in 2.5 cm root trainers filled with 1:1:1 sand, soil and FYM sterilized mixture. Root trainers with transplanted plants were placed under 30±2°C and 60±5% RH for 15-20 days in a glass house for acclimatization. Finally, acclimatized plants were transferred to field.

Three separate experiments were laid out in two factors factorial experiment in Completely Randomized Design with two replications and two factors of genotypes and different culture media combination. Per replication approximately 100-120 explants were excised and cultured on each media. The arc-sine transformation was made before the analysis of data, since all data were calculated in percentage. The data were analyzed as per method suggested by Snedecor and Cochran (1967).

Results and discussions

The basic goal of *in vitro* culture of *Withania* was to develop reliable protocol for achieving plant regeneration in higher frequencies from diverse explants cultures in order to use them for mass *in vitro* propagation of desirable genotypes/cultivars or elite clones and advance biotechnological works. Although theoretically each and every somatic cell of a plant is totiopotent, callus induction and regeneration frequency of a cell is influenced by several factors like species, nature of explants and *in vitro* culture conditions like establishment of aseptic culture, nutrientional composition of basal medium, types and concentrations of plant growth regulators added in medium and other culture conditions. The degree of growth and differentiation varied considerably with the nutritional composition of basal medium (Sekhawat *et al*., 1993). During present investigation basal MS medium was used throughout the experiment, as this was found more responsive as compared to MS in course of preliminary experiments. The need of MS salt for shoot multiplication showed the moderate salt requirement for the growth of *Withania somnifera* in present study. Various other scientists (Sen and Sharma, 1991; Rani and Grover, 1999; Kulkarni *et al*., 2000; Manickam *et al*., 2000; Govindraju *et al*., 2003; Rani *et al*., 2003; Sivanesan and Muragesan, 2008; Sharma *et al*., 2010) also used MS basal medium for culturing of diverse explants of *Withania*.

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Mature embryo, cotyledon and hypocotyl explants of two *Withania* cultivars were cultured on various fortifications of MS medium. During the present investigation, cultured explants followed mostly indirect pathways of plantlet regeneration i.e. plantlets originated *via* callus formation either indirect somatic embryogenesis or organogenesis. The first response of cultured explants was similar after 5-7 days and generally independent from explants and culture media. All explants became swollen and no callus proliferation was apparent during first few days. After 5-7 days of culture, callus initiation was observed from the most of the explants. Callus initiation was usually started from the cut edges from cultured mature cotyledons (Fig.1A), whereas, from mature embryos, callus formation was observed from embryonic axis (Fig.1B). In hypocotyl segment, profuse callus growth usually started from the wounded ends and spread towards the middle region of the tissue segment (Fig.1C). Beginning of callus initiation from cut ends of cultured mature cotyledon and hypocotyls was probably due to more absorption of nutrients leading to rapid cell division and subsequent callus formation. This finding is in accordance with Sharma *et al*. (2010) for *Withania* leaf explant culture. Induced calli were light to moderate yellow and greenish in color, small to medium in size and friable to compact in texture.

In cultured mature cotyledons, embryoid formation started after approximately 10 days from initial culturing. The embryoid like structures were rounded with irregular outlines usually appeared in clusters (Fig.1D). Such somatic embryos germinated after transfer into regeneration medium (Fig.1 E). In mature embryos, embryoid like structures developed on callus surface (Fig.1 F). Gradually, these structures developed into multiple shoots (Fig.1G-H). In cultured hypocotyls, shootlets developed from the nodules arising on the surface of the callus (Fig. 1I-J). Shoot formation started approximately 10 days from initial culturing. However, the duration varied from culture to culture and in a few cases shoots formed after 45 days. Most of the calli, after prolonged culturing on the induction media gave rise to plantlets. However, transfer into regeneration medium allowed higher growth rate and elongation (Fig 1.K). Various shoot forming calli were able to produce one or many plantlets at a time. Complete plantlets regenerated *via* embryogenesis and shoots developed *via* organogenesis were counted as regenerated plantlets. Regenerated shoots alone were also counted as plantlets since they gave rise to complete plants after rhizogenesis on rooting medium**.** Later these regenerants were transferred to the Net House for 25-30 days (Fig.1L) for acclimatization. The plants, after survival in the net house conditions, were evaluated visually on the basis of

their appearance. Although the traits were not scored quantitatively, regenerated plants were found phenotypically normal and true to the type.

The analysis of variance presented in Table 1-3 revealed that there were highly significant ($p<0.01$) differences exist among the response of genotypes, culture media combinations and their interactions in terms of overall callus induction, formation of mrophogenic calli and plantlet regeneration. It indicates the presence of considerable amount of variability amongst the different culture media combinations, genotypes as well as their interactions.

In cultured mature cotyledons and embryos, higher callus induction was witnessed on induction medium MS2D.5B (76.54% and 77.68% respectively) closely followed by culture medium MSD.5B (72.63% and 74.22% respectively), while, for cultured hypocotyl segments, initiation media MS2N.5Kn (70.56%) and MSN.5Kn (68.75%) followed by MS3N.5B (70.56%) proved superior for higher degree callus induction. However, poor callus induction was observed with application of lower concentration of 2,4-D and NAA (0.1-0.5 $mg.l^{-1}$) either alone or in combination with 0.5 $mg.l^{-1}$ BA. Furthermore, the frequency of callus initiation also decreased at higher concentration of 2,4-D (≥3.0-5.0 $mg.l^{-1}$) due to mortality and eventually death of cells. NAA when used at higher concentration ((≥3.0-5.0 $mg.l^{-1}$) formed roots along with small amount of callus. Present findings reveal that culture medium fortified with relatively higher concentrations of auxins (2,4-D or NAA) in combination with lower concentration of cytokinin (BAP/ Kn) initiated calli in higher frequencies. Higher degree of callus initiation was observed on culture media MS2D.5B/ MSD.5B and MS2N.5Kn/ MSN.5Kn as compared to culture media MS5D.5B/ MS5N.5B and MS5N.5Kn containing very higher concentration of an auxin in combination with lower concentration of cytokinin and MS5B.5N / MS5Kn.5N supplemented with relatively higher concentration of cytokinin in combination with lower concentration of an auxin suggested that relatively higher concentration of an auxin in conjunction with comparatively lower concentration of a cytokinin is adequate for higher degree of callus initiation for diverse explants cultures in *Withania*. Similar results were also reported by Rani and Grover (1999), Govindraju *et al*. (2003) and Sharma *et al*. (2010) in *Withania*.

From cultured mature cotyledon and embryo explants, maximum morphogenic calli was formed by a group of three similar responding culture media combinations, *namely:* MS2B.5N (40.84%, 43.52%), MSB.5N (40.62%, 42.58%) and MS.5B.5N (40.27%, 42.40%) respectively. However, in cultured hypocotyl only medium MS2B.5N (35.67%) supported higher morphogenic potential. These findings revealed that all explants formed morphogenic calli in higher frequencies when culture media amended with cytokinin BAP in equal

or relatively higher concentrations in combination with lower concentration of an auxin NAA. Moreover, higher morphogenic calli formation rate was evidenced on culture media MS2B.5N/MSB.5N as compared to MS5D.5B/ MS5N.5B that suggested that higher concentration of an auxin in combination with a lower concentration of a cytokinin is not necessary for this purpose. Much lower results with 2,4-D, NAA or BAP alone on Media MS5D, MS5N or MS5B (even in higher concentrations) as compared to combination of NAA with BAP revealed that a cytokinin as well as an auxin as sole were insufficient for higher degree of morphogenic callus formation. Regenerants in higher numbers were also recovered from culture media MS2B.5N (> 83% from cultured mature cotyledon and embryo and ~70% from cultured hypocotyl) and MSB.5N (> 79% from cultured mature cotyledon and embryo and ~69% from cultured hypocotyl). These findings revealed that diverse explants cultures regenerated plantlets in higher frequencies when culture media amended with relatively higher concentrations of BAP in combination with lower concentration of an auxin NAA. Reports of earlier scientists (Kulkarni *et al*., 2000; Govindraju *et al*., 2003; Sivanesan *et al*., 2007; Sharma *et al*., 2010) on *in vitro* morphogenesis in diverse explants cultures of *Withania* on the same hormonal regime (NAA and BAP) supported the results obtained by the authors.

In vitro produced shoots were treated with GA_3 at 1.0 mg l^{-1} gave the maximum shoot length. However, further increase in the concentration of GA_3 ($\geq$ 1.0 mg.l^{-1}) decreased the elongation of shoots (data not presented). This finding is in accordance with findings of Sivanesan (2007) in *W. somnifera*. Elongated shoots were then subjected to different concentration of diverse auxins (IBA, NAA and IAA) for root induction. MS basal medium containing 2.0 mg l^{-1} IBA was found to be the best. It produced >90% rooting (data not shown). An increase in concentration above 2.0 mg.l^{-1} IBA reduced the root induction and promoted callusing. Similar results also addressed by Rani and Grover (1999), Anjali *et al*., (2000), Manickam *et al*., (2000), Rani *et al*., (2003), Sivanesan (2007) and Sharma *et al*., (2010) in *W. somnifera* . Regenerated plantlets were transferred to the field with more than 80% of survival rate after hardening in Green House for 25 days followed by for 30 days in Net House.

Influences of various factors on *in vitro* response indicated that something within an explant is as critical for a given response as its genotype. Many of the genetic differences could be circumvented by the growing source of plants under optimal conditions and also by varying nutrients and plant growth

regulators supplemented into the culture medium. During present investigation, all explants, *namely*: mature cotyledon and embryo and hypocotyls were obtained from germinating seeds or seedling developed under lab/controlled conditions, influence morphogenic and plant regeneration ability up to a great extent. It seems reasonable to conclude that variations obtained for *in vitro* response resulted from the genetical differences among genotypes. During present investigations considerable variability for *in vitro* response was observed in both genotypes for various explant cultures. In general, JA-20 responded well than MWS-100, for the most of the explant cultures for the most of the culture phases. In *Withania*, genotypic differences have also been reported for various explants cultures by Rani and Grover (1999), Rani *et al.,* (2000) and Govindraju *et al*., (2003).

It is well known fact that morphogenic response in tissue culture is affected by the type of explants (Lazer *et al*., 1990). For regeneration ability, various explants responded differently. Maximum numbers of shoots were recovered from mature embryo followed by explants mature cotyledon and hypocotyl. Varying responses in culture among diverse explants cultures were also observed by Rani and Grover (1999) and Govindraju *et al*., (2003) in *Withania*. These findings are suggested that explants with higher regeneration potential can be used for genetic transformation and other biotechnological work.

In addition to genotypic and culture medium influenced on all phases of diverse explants cultures of *Withania*, strong genotype x culture medium interactions for the most of the culture phases were also observed. Genotypes on either MS2D.5B or MSD.5B or in both nutrient media induced more than 72 per cent callus from mature cotyledon and embryo explants cultures. Interactions of the both of the genotypes with induction medium MS2N.5Kn and MSN.5Kn also exhibited similar results for formation of callus from cultured hypocotyl segments. For formation of morphogenic calli and regeneration of plantlets, either culture medium MS2B.5N or MSB.5N or both were found more responsive (more than 40% morphogenic calli and more than 80% plantlets were regenerated) from the both genotypes. This suggests that a particular genotype selected for advance work can be cultured on the most suitable medium to obtain maximum response. Also, the possibility exists for improvement of *in vitro* efficiency of a particular genotype by further modifying the culture medium.

During the present investigation, we used the novel technique in mature cotyledon, embryo and hypocotyl cultures, which could be obtained from mature seed. However, many workers used immature seed to obtain the explant embryo and cotyledon. Since Ashwagandha is propagated mainly by seed and

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the success rate of vegetative propagation being very low, this rapid and efficient regeneration protocol could be used for large-scale multiplication of selected cultivated varieties. As the number of shoots produced per explant was substantially higher in these explants. Callus cultures can be exploited further for enhancing many important secondary metabolites of *Withania* especially from roots by elicitation and immobilization techniques. These techniques may also be employed for genetic transformation studies using *Agrobacterium tumefaciens* and *A. rhizogenes*.

Figure 1. Plant regeneration from diverse explant cultures in *Withania*: **A.** Mature cotyledons after 7-10 days in culture; **B.** Mature embryos after 7-10 days in culture; **C.** Mature hypocotyls after 7-10 days in culture; **D.** Initiation of somatic embryos from cultured mature cotyledons; **E.** Germination of somatic embryos; **F.** Initiation of somatic embryos from cultured mature embryos**; G.** Germination of somatic embryos; **H**. Regeneration of multiple plants from cultured mature embryos; **I-J.** Regeneration of multiple plantlets from cultured hypocotyls; **J**. Elongated shootlets after 35-40 days in culture; **K**. Hardening of regenerant in Net House.

Table 1. Combined effect of different added auxins and cytokinins in varying concentrations and combinations on *in vitro* response of cultured mature cotyledons.

Culture Media ▼	Plant Growth regulat mg. l^{-1}			Callus initiation (%)			Morphogenic Calli (%)			Plant regeneration (%)		
Genotypes	NAA	BAP	2, 4-D	JA-20	MWS-10(	Mean	JA-20	MWS-10(	Mean	JA-20	MWS-	Mean
MS.1N.5B	0.1	0.5	-	36.75	32.70	34.72^{l}	24.69	16.61	20.65^{l}	36.94	32.90	34.92^{h}
MSN.5B	1.0	0.5	-	64.71	59.66	62.18^{de}	32.56	28.50	30.53^{c}	43.15	39.09	41.12^{g}
MS2N.5B	2.0	0.5	-	65.72	62.56	64.14^{d}	32.50	28.46	30.48^{c}	57.16	51.11	54.13^{e}
MS3N.5B	3.0	0.5	-	63.62	57.56	60.59^{e}	31.13	25.07	28.10^{c}	55.19	49.14	52.16^{f}
MS4N.5B	4.0	0.5	-	48.71	44.67	46.69^{hi}	29.95	23.89	26.92^{c}	53.31	46.27	49.79^{f}
MS5N.5B	5.0	0.5	-	45.75	39.69	42.72^{j}	19.41	15.37	17.39^{l}	38.36	34.32	36.34^{h}
MS.1D.5B	-	0.5	0.1	46.88	42.84	44.86^{i}	19.54	15.48	17.51^{l}	31.02	22.01	26.51^{i}
MS.5D.5B	-	0.5	0.5	53.75	47.70	50.72^{g}	21.96	17.92	19.94^{l}	27.62	23.58	25.60^{ij}
MSD.5B	-	0.5	1.0	74.65	70.61	72.63^{b}	22.88	18.84	20.86^{l}	25.21	23.41	24.31^{j}
MS2D.5B	-	0.5	2.0	78.56	74.52	76.54^{a}	24.35	20.29	22.32^{c}	25.64	21.60	23.62^{j}
MS3D.5B	-	0.5	3.0	68.80	64.76	66.78^{c}	21.45	17.39	19.42^{l}	24.86	16.78	20.82^{k}
MS4D.5B	-	0.5	4.0	57.84	53.80	55.82^{f}	19.40	15.36	17.38^{l}	14.13	12.25	13.19^{l}
MS5D.5B	-	0.5	5.0	33.85	29.81	31.83^{m}	17.69	13.75	15.72^{l}	13.52	9.48	11.50^{l}
MS.1B.5N	0.5	0.1	-	50.56	44.50	47.53^{h}	37.67	35.72	36.69^{b}	69.68	55.62	62.65^{d}
MS.5B.5N	0.5	0.5	-	46.68	42.64	44.66^{i}	42.30	38.25	40.27^{a}	75.15	69.11	72.13^{c}
MSB.5N	0.5	1.0	-	45.69	41.65	43.67^{j}	42.60	38.64	40.62^{a}	81.76	77.12	79.44^{b}
MS2B.5N	0.5	2.0	-	44.75	38.69	41.72^{k}	42.36	39.32	40.84^{a}	84.84	82.80	83.82^{a}
MS3B.5N	0.5	3.0	-	41.72	37.68	39.70^{k}	36.28	34.22	35.25^{b}	75.71	69.63	72.67^{c}
MS4B.5N	0.5	4.0	-	16.85	10.79	13.82^{n}	25.60	21.68	23.64^{c}	68.15	62.09	65.12^{d}
MS5B.5N	0.5	5.0	-	12.82	8.78	10.80^{o}	19.40	15.36	17.38^{l}	50.20	54.14	52.17^{ef}
Mean				46.00^{a}	45.28^{b}		28.18^{a}	24.00^{b}		47.58^{a}	42.62^{b}	
CD 0.05												
Genotypes						0.96			1.05			0.96
Media						2.61			2.83			2.60
G x M						4.52			4.90			4.51

Values within column followed by different letters are significantly different at 5% probability level.

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Table 2. Combined effect of different added auxins and cytokinins in varying concentrations and combinations on *in vitro* response of cultured mature embryos.

Culture Media	Plant Growth Regulators mg.l^{-1}				Callus initiation (%)			Morphogenic Calli (%)			Plant regeneration (%		
▼ Genotypes	NAA	TDZ	BAP	2, 4-D	JA-20	MWS-100	Mean	JA-20	MWS-	Mean	JA-20	MWS 100	Mean
MS.1N.5T	0.1	0.5	-	-	37.90	34.82	**36.36^{l}**	20.45	16.41	**18.43^{j}**	38.50	34.46	**36.48^{jk}**
MS.5N.5T	0.5	0.5	-	-	44.12	40.08	**42.10^{k}**	23.07	19.03	**21.05^{i}**	42.58	38.54	**40.56^{i}**
MSN.5Td	1.0	0.5	-	-	65.82	62.75	**64.28^{d}**	34.16	30.12	**32.14^{d}**	66.74	62.70	**64.72^{e}**
MS2N.5Td	2.0	0.5	-	-	68.67	64.63	**66.65^{c}**	42.20	38.16	**40.18^{b}**	70.42	66.38	**68.40^{d}**
MS3N.5Td	3.0	0.5	-	-	69.86	66.76	**68.31^{c}**	44.23	40.41	**42.32^{a}**	66.49	62.43	**64.46^{e}**
MS4N.5Td	4.0	0.5	-	-	50.13	46.09	**48.11^{gh}**	40.26	36.22	**38.24^{b}**	62.71	58.65	**60.68^{f}**
MS5N.5Td	5.0	0.5	-	-	48.72	39.80	**44.26^{j}**	24.15	20.11	**22.13^{hi}**	40.50	36.42	**38.42^{j}**
MS.1D.5B	-	-	0.5	0.1	50.16	45.09	**47.62^{h}**	25.33	21.29	**23.31^{gh}**	54.90	50.86	**52.88^{h}**
MS.5D.5B	-	-	0.5	0.5	54.70	50.66	**52.68^{f}**	28.36	24.32	**26.34^{f}**	58.18	54.14	**56.16^{g}**
MSD.5B	-	-	0.5	1.0	76.25	72.20	**74.22^{b}**	36.42	32.38	**34.40^{c}**	58.14	54.10	**56.12^{g}**
MS2D.5B	-	-	0.5	2.0	80.20	75.17	**77.68^{a}**	34.13	30.09	**32.11^{c}**	40.46	36.42	**38.44^{ij}**
MS3D.5B	-	-	0.5	3.0	60.19	56.15	**58.17^{e}**	33.39	29.35	**31.37^{d}**	38.14	34.10	**36.12^{k}**
MS4D.5B	-	-	0.5	4.0	46.78	59.72	**53.25^{f}**	31.10	27.05	**29.07^{e}**	36.60	32.56	**34.58^{kl}**
MS5D.5B	-	-	0.5	5.0	35.14	31.09	**33.11^{l}**	18.57	14.53	**16.55^{j}**	34.84	30.80	**32.82^{l}**
MS.1B.5N	0.5	-	0.1	-	50.89	46.85	**48.87^{g}**	41.66	37.62	**39.64^{b}**	74.70	70.66	**72.68^{c}**
MS.5B.5N	0.5	-	0.5	-	48.21	44.17	**46.19^{hi}**	44.42	40.38	**42.40^{a}**	78.50	74.46	**76.48^{b}**
MSB.5N	0.5	-	1.0	-	46.89	42.85	**44.87^{ij}**	44.60	40.56	**42.58^{a}**	84.29	80.23	**82.26^{a}**
MS2B.5N	0.5	-	2.0	-	45.12	41.08	**43.10^{jk}**	45.54	41.50	**43.52^{a}**	85.97	81.91	**83.94^{a}**
MS3B.5N	0.5	-	3.0	-	43.14	39.10	**41.12^{k}**	40.18	36.14	**38.16^{b}**	80.78	76.74	**78.76^{b}**
MS4B.5N	0.5	-	4.0	-	16..94	12.90	**14.92^{m}**	26.91	22.87	**24.89^{fg}**	75.84	71.80	**73.82^{c}**
MS5B.5N	0.5	-	5.0	-	14.77	10.79	**12.78^{m}**	20.32	16.28	**18.30^{j}**	71.68	67.64	**69.66^{d}**
Mean					**51.07^{a}**	**46.79^{b}**		**33.32^{a}**	**29.37^{b}**		**60.04^{a}**	**55.99**	
CD 0.05													
Genotypes							**0.82**			**0.78**			**0.81**
Media							**2.23**			**2.11**			**2.20**
G x M							**3.85**			**3.66**			**3.82**

Values within column followed by different letters are significantly different at 5% probability level

Table 3. Combined effect of different added auxins and cytokinins in varying concentrations and combinations on *in vitro* response of cultured mature hypocotyls.

Culture Media ▼	Plant Growth Regulators mg. l^{-1}			Callus initiation (%)			Morphogenic calli (%)			Plant regeneration (%)		
Genotypes►	NAA	BAP	Kineti	JA-20	MWS-1(	Mean	JA-2(	MWS-10(	Mean	JA-20	MWS-1(	Mean
MS.1N.5B	0.1	0.5	-	34.10	28.08	**31.09**^{i}	23.24	19.20	**21.22**^{i}	56.15	46.05	**51.10**^{e}
MS.5N.5B	0.5	0.5	-	45.68	39.62	**42.65**^{h}	36.19	28.11	**32.15**^{l}	61.41	55.35	**58.38**^{c}
MSN.5B	1.0	0.5	-	64.30	56.24	**60.27**^{c}	35.41	29.35	**32.38**^{l}	73.45	62.35	**67.90**^{b}
MS2N.5B	2.0	0.5	-	65.54	59.49	**62.51**^{c}	36.81	28.73	**32.77**^{l}	70.62	62.35	**66.48**^{b}
MS3N.5B	3.0	0.5	-	68.73	59.63	**64.18**^{b}	35.15	27.07	**31.11**^{l}	64.72	56.64	**60.68**^{c}
MS4N.5B	4.0	0.5	-	45.72	39.66	**42.69**^{h}	21.17	15.11	**18.14**^{i}	65.31	59.25	**62.28**^{c}
MS5N.5B	5.0	0.5	-	44.16	36.06	**40.11**^{h}	21.68	12.59	**17.13**^{i}	35.47	25.37	**30.42**^{g}
MS.1N.5Kn	0.1	-	0.5	47.91	37.81	**42.86**^{g}	23.38	17.35	**20.36**^{i}	38.42	26.34	**32.38**^{g}
MS.5N.5Kn	0.5	-	0.5	52.77	44.69	**48.73**^{f}	27.58	19.50	**23.54**^{i}	43.55	33.45	**38.50**^{f}
MSN.5Kn	1.0	-	0.5	72.79	64.71	**68.75**^{a}	33.61	23.51	**28.56**^{i}	53.84	45.76	**49.80**^{e}
MS2N.5Kn	2.0	-	0.5	75.61	65.51	**70.56**^{a}	33.86	25.78	**29.82**^{l}	37.45	27.35	**32.40**^{g}
MS3N.5Kn	3.0	-	0.5	58.63	52.57	**55.60**^{d}	23.75	17.69	**20.72**^{i}	34.12	26.04	**30.08**^{h}
MS4N.5Kn	4.0	-	0.5	57.82	47.79	**52.80**^{e}	20.67	10.57	**15.62**^{i}	31.43	25.37	**28.40**^{h}
MS5N.5Kn	5.0	-	0.5	33.82	27.86	**30.84**^{i}	11.71	5.65	**8.68**^{g}	31.77	21.67	**26.72**^{h}
MS.1B.5N	0.5	0.1	-	47.86	39.78	**43.82**^{g}	32.39	26.33	**29.36**^{i}	55.65	47.52	**51.58**^{e}
MSB.5N	0.5	1.0	-	46.77	36.67	**41.57**^{h}	38.47	28.38	**33.42**^{l}	74.34	64.24	**69.29**^{a}
MS2B.5N	0.5	2.0	-	45.75	35.65	**40.70**^{h}	38.73	32.62	**35.67**^{i}	73.93	67.87	**70.90**^{a}
MS3B.5N	0.5	3.0	-	41.63	35.57	**38.60**^{g}	30.69	26.65	**28.67**^{i}	72.80	64.72	**68.76**^{a}
MS4B.5N	0.5	4.0	-	16.76	6.77	**11.76**^{j}	28.76	20.68	**24.72**^{i}	70.88	60.78	**65.83**^{b}
MS5B.5N	0.5	5.0	-	12.83	6.77	**9.80**^{j}	13.55	7.49	**10.52**^{l}	65.59	59.53	**62.56**^{c}
Mean				**48.95**^{a}	**41.04**^{b}		**28.34**	**21.11**^{b}		**54.54**^{a}	**46.90**^{b}	
CD 0.05												
Genotypes						**1.76**			**1.92**			**1.50**
Media						**4.76**			**5.21**			**4.05**
G x M						**8.25**			**9.03**			**7.02**

Values within column followed by different letters are significantly different at 5% probability level.

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