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In vitro regeneration study in lablab bean and dolichos bean (*Lablab purpureus* (L). Sweet) Genotypes

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Abstract
The current investigation was carried out to study *in vitro* regeneration potential in Lablab bean genotypes and Dolichos bean Cv. (Konkan Bhushan). In surface sterilization, treatment with 70% alcohol for 5 minute followed by $HgCl_2$ (0.1%) for 10 minutes was found the most effective in establishing aseptic cultures (97.22%). Mature embryo axis with single cotyledon (MEASC) explant on medium MS+ 6 mg/l BAP+ 0.5 mg/l NAA responded well for shoot induction frequency, multiple shoot induction in Dolichos bean genotype 'Konkan Bhushan'. The medium MS+ 0.5mg/l BAP+ 0.2 mg/l GA_3 was found better for elongation of *in vitro* regenerated shoots. The genotype 'Konkan Bhushan' rooted also earliest in 9.30 days on medium MS+ 1.0 mg/l IBA+ 2% sucrose with highest root induction frequency (79.51%) and maximum roots per shoot (5.04). During hardening, the potting mixture of soil, vermiculite and sand in 1:1:1 showed the highest survival rate.

Keywords: *In vitro* regeneration, surface sterilization, mature embryo axis with single cotyledon (MEASC)

Introduction
Lablab bean (*Lablab purpureus* (L) Sweet var. typicus), also known as Dolichos bean, hyacinth bean, field bean, is popularly known as Val in Maharashtra state. It is member of family fabaceae, having chromosome number 2n=22. In India, it is grown mainly in Maharashtra, Utter Pradesh, Madhya Pradesh, Tamil Nadu, Andhra Pradesh and Karnataka. In Konkan region, it is grown in Thane, Palghar, Raigad, Ratnagiri and Sindhudurg districts on residual moisture. Lablab bean has the capacity to fix atmospheric nitrogen in the soil and it can do relatively well even under poor soil fertility conditions since it is leguminous crop. Lablab bean yield is limited because of biotic and abiotic factors especially pathogenic, entomological, agronomical, genetic etc. The lower productivity of crop is attributed to many factors, among them pest is major factor. Conventional breeding method imparting resistance to such stresses is having many limitations like presence of proteolytic enzymes, hairs on plant flower, time period. Conventional method is heritably safe but is slow and time consuming. To accelerate the breeding programme genetic engineering is a better option through which gene could be transferred from any source to variable host giving rise to transgenic crops. The ability to regenerate plants from cultured cells, tissues or organs constitutes the basis for producing transgenic crops. The regeneration potential depends on the genotype. Besides this, as there is lot of importance of the dolichos bean in agricultural economy of the Konkan region, it is necessary to apply tissue culture technique in agricultural research programme. Therefore the present study was carried out to study *in vitro* regeneration potential of different Lablab bean genotypes and Dolichos bean Cv. (Konkan Bhushan).

Material and Methods
In the present investigation the 10 genotype of Lablab bean were collected from Agricultural Botany Farm of Dr. Balasaheb Konkan Krishi Vidyapeeth, Dapoli and used for *In Vitro* regeneration. The seeds of popular genotype of Dolichus Bean "Konkan Bhushan" were collected from Vegetable Improvement Scheme, Central Experimental Station, Wakawali (Table 5). Prior to regeneration, surface sterilization protocol was standardized. Different sterilization treatments consisting of ethanol and mercuric chloride ($HgCl_2$) of different concentrations were tested for varied time periods (Table 4).
For regeneration experiment, 3 explants E_1, E_2 and E_3 i.e. mature embryo axes (MEA), mature embryo axes with single cotyledon (MEASC) and shoot tip, respectively were used. The surface sterilized seeds were soaked for 15-16 hours and then removed seed coat and mature

levels of BAP, kinetin, NAA, under controlled condition for multiple shoot induction (Table 1). Observations were recorded on regeneration frequency, days to shooting and number of shoots per explant.

Table 1: Media combinations used for multiple shoot induction

Tr. No.	Treatment details
SIM_1	MS medium (SIM control)
SIM_2	MS + 2 mg/l BAP
SIM_3	MS + 4 mg/l BAP
SIM_4	MS + 6 mg/l BAP
SIM_5	MS + 2 mg/l BAP + 0.5 mg/l NAA
SIM_6	MS + 4 mg/l BAP + 0.5 mg/l NAA
SIM_7	MS + 6 mg/l BAP + 0.5 mg/l NAA
SIM_8	MS + 2 mg/l kinetin
SIM_9	MS + 4 mg/l kinetin
SIM_{10}	MS + 6 mg/l kinetin

The shoots induced were then inoculated on different media combinations for shoot elongation (Table 2) and observations regarding 'per cent elongation response' and 'no. of shoots elongated per explant' were recoeded.

Table 2: Media combinations used for shoot elongation

Tr. No.	Treatment details
SEM_1	MS media (SEM control)
SEM_2	MS + 0.5 mg/l kinetin + 0.2 mg/l GA_3
SEM_3	MS + 1.0 mg/l kinetin + 0.2 mg/l GA_3
SEM_4	MS + 2.0 mg/l kinetin + 0.2 mg/l GA_3
SEM_5	MS + 3.0 mg/l kinetin + 0.2 mg/l GA_3
SEM_6	MS + 0.5 mg/l BAP + 0.2 mg/l GA_3
SEM_7	MS + 0.2 mg/l GA_3
SEM_8	MS + 0.5 mg/l GA_3

Elongated shoots were separated and further inoculated on rooting media combinations (Table 3) to observe days to rooting, per cent rooting (%) and number of roots per plantlet.

Table 3: Media combinations for root induction

Tr. No.	Treatment details
RIM_1	MS + 0.1 mg/l NAA
RIM_2	MS + 0.5 mg/l NAA
RIM_3	MS + 1.0 mg/l NAA
RIM_4	MS + 0.5 mg/l IBA
RIM_5	MS + 1.0 mg/l IBA
RIM_6	MS + 0.5 mg/l IBA + 2 % sucrose
RIM_7	MS + 1.0 mg/l IBA + 2 % sucrose
RIM_8	MS + 1.0 mg/l NAA + 0.5 mg/l BAP
RIM_9	MS + 0.5 mg/l NAA + 0.5 mg/l BAP

The individual plantlets were transferred to potting mixture containing soil, vermiculite, sand with different proportion (Table) and covered with plastic bag with small hole for air circulation and kept in growth chamber. For the initial 10-15 days high humidity was maintained (>90%) and gradually reduced to the ambient level over a period of 2 to 4 weeks. The experiment in 3 replicates was carried out. The culture conditions were maintained at 25± 2 °C temperature, light intensity of 1000-2000 lux with a light and dark cycle of 16 and 8 hours. Factorial-Completely Randomized Design (F-CRD) was used for statistical analysis.

Results and Discussion

Surface sterilization

Contamination with microorganisms is considered to be the single most important reason for losses during the *in vitro* regeneration system of plants. During sterilization the living materials should not lose their biological availability and any contaminants should be eliminated, therefore explants are surface sterilized only by treatment with disinfectant solution at suitable concentration for a specified period. Among the nine treatments with various combinations tried, the treatment T_6 consisting of 70% alcohol for 5 minutes followed by $HgCl_2$ immersion @ 0.1% for 10 minutes recorded maximum (97.22%) aseptic culture establishment without affecting the explant adversely (Table 4). Therefore it could be regarded as the effective surface sterilization treatment. It has been also observed that increased duration of alcohol treatment was detrimental effect on explant. The lower concentration of $HgCl_2$ is always beneficial since it causes carcinogenic effect after certain limit. It is reported that at higher concentration, all sterilizing agents showed maximum effect against microbiological contamination, although the survival percentage was low. Oyebanji *et al*. [2] reported that increased duration with higher concentration of alcohol resulted into developmental contamination and this could be due to decay of explant. This study indicates that there is need of optimum concentration and duration of sterilization explant to get aseptic cultures.

Table 4: Surface sterilization treatments for aseptic culture establishment

Tr. No.	Treatment details	Time (min)	Per cent aseptic cultures (%)
T_1	Control (DDW washing)	10	0.00
T_2	70 % alcohol	10	95.55
T_3	$HgCl_2$ (0.1%)	10	28.89
T_4	$HgCl_2$ (0.5%)	5	42.22
T_5	$HgCl_2$ (1.0%)	5	82.22
T_6	70% alcohol+ $HgCl_2$ (0.1%)	5+10	97.22
T_7	70% alcohol+ $HgCl_2$ (0.5%)	10+10	86.67
T_8	70% alcohol+ $HgCl_2$ (1.0%)	10+5	17.78
T_9	70% alcohol+ $HgCl_2$ (1.0%)	10+10	68.89
	Range	Minimum	0.00
		Maximum	97.22

	SEm±		2.34
	CD at 1 %		9.54
	Significant at 1 %		Sig

Direct regeneration (*in vitro*)

The direct regeneration through induction of adventitious shoots using meristematic tissues and complete regeneration other hand became popular as there are no reports of genetic variation in progeny [3]. Almost all reports of regeneration in legumes have been confined to members of the papilionoidae, the sub family of great economical important plants had been regenerated via organogenesis from 54 different legume species while only 39 species has been shown regeneration from somatic embryos [4].

Recalcitrance of crop is attributed to genetic differences that block the regeneration process in tissue culture. Morphogenic response of many legumes have been shown to be under genetic background [5]. So in several crops different cultural conditions were found to be necessary for regeneration of different genotypes. Similarly plant regeneration from different tissues is of great importance in obtaining transgenic individual. The explants also had highly significant effect on per cent plantlet regeneration. The shoot-tip explant recorded higher regeneration response than cotyledonary node explants [6]. The regeneration of shoot buds from various explants of pigeon pea such as leaves [7] has been reported. Similarly, media combination is also greatly influenced on regeneration ability of explants. Naz *et al*. [8] reported that the regeneration frequency increased with increase in concentration of cytokinins.

In present study, nine genotypes of lablab bean and one popular genotype of dolichos bean *Cv*. Konkan bhushan were employed for the regeneration studies. The frequency of shoot formation was influenced by the type of explants. The study conducted by Kale V. P. [6] revealed that shoot tip had grater regeneration potential than cotyledonary explant in field bean. But in present study mature embryo axis with single cotyledon showed maximum frequency of shoot induction followed by mature embryo axis and shoot tip. Similar observations were also made in *Phaseolus vulgaris* by Mallik and saxena [5].

In the present study, there was significant difference in genotype for shoot induction. Among the various genotypes studied, Konkan Bhushan recorded maximum (96.00%) regeneration frequency (Table 5). The study conducted by Kale V. P. [6] recorded 92% regeneration response in Konkan Wal. It indicates that regeneration response depends upon endogenous level of cytokynin in different genotypes. The variety Konkan Bhushan also showed maximum response for multiple shoot induction and recorded 11.33 multiple shoots (Table 6). The genotypic variation in shoot induction studies in many pulses confirmed these results.

Organogenic differentiation in cell and tissue cultures is due to hormonal manipulation in culture medium. The role of exogenous cytokinin during induction phase is difficult to assess. More commonly used cytokinins are BAP, kinetin, TDZ and Zeantin. In this study response of shoot induction was positively correlated with increased dose of BAP (6 mg/l) and NAA (0.5 mg/l).

In contrast to this reduction in number of shoots with increasing concentration of BAP and NAA was reported by Franklin *et al.* [9] In this study the optimum dose of BAP was worked for shoot induction.

Interestingly *in vitro* culture of MEASC in presence of BAP was found to be beneficial and induced 11.33 shoots per explant in Konkan Bhushan within 6.21 days (Table 7). Experience of this study indicates the potential of BAP in combinations with NAA as an indicative signal for shoot initiation and usefulness of MEASC as a tool to induced morphogenesis in lablab bean. This study confirms the results obtained by Khatun *et al.* [10]

Shoot elongation

Generally, giberllic acid gives good result for shoot elongation with 3 to 5 days. Reduction in cytokinin levels in regeneration medium favors proper elongation of shoot buds because, it exerts less phytohormonal stress during plant development [11]. A good growth of the shoot is especially critical when shoot multiplication through axillary branching achieved by taking nodal segments at each subculture. In such cases the rate of shoot multiplication is directly related to the elongation of the shoots and the number of nodal cutting available at the end of each passage. In pigeon pea GA_3 (2.9 µM) improved the rate of multiplication, growth and quality shoots. In the present study cytokinin like BAP was found to be much effective at lower concentration (MS + 0.5 mg/l BAP) and gave 100% shoot elongation response (Table 8). But, at high concentration the response to shoot elongation was reduced. Lower level of IAA (MS + 0.5 mg/l BAP) also favoured elongation of shoots (13.33%). Franklin *et al.* [9] (1998) obtained proper elongation of shoots on MS medium with low levels of BAP. In present study, 15.00 and 2.00 shoots were elongated per plant at the lower concentration of kinetin (MS+0.5 mg/l kinetin) and IAA (MS+ 0.5 IAA), respectively.

Table 5: Effect of Genotype, Explant and Medium on shoot induction frequency in in vitro regeneration

Genotypes		SIM_1	SIM_2	SIM_3	SIM_4	SIM_5	SIM_6	SIM_7	SIM_8	SIM_9	SIM_{10}
G_1 (No. 47)	E_1	45.33 (42.3)	57.33 (49.20)	61.33 (51.54)	58.67 (49.97)	64.00 (53.13)	69.33 (56.36)	70.67 (57.20)	54.67 (47.66)	52.00 (46.13)	50.67 (45.37)
	E_2	38.67 (38.43)	57.33 (49.20)	62.67 (52.32)	66.67 (54.75)	68.00 (55.56)	74.67 (59.77)	82.67 (65.40)	50.67 (45.36)	42.67 (40.76)	36.00 (36.80)
	E_3	32.00 (34.44)	41.33 (39.99)	46.67 (43.07)	48.00 (43.84)	56.00 (48.43)	53.33 (46.89)	49.33 (44.60)	36.00 (36.86)	38.67 (38.43)	44.00 (41.54)
G_2 (No. 15)	E_1	53.33 (46.89)	65.33 (53.92)	68.00 (55.53)	66.67 (54.72)	72.00 (58.07)	77.33 (61.56)	78.67 (62.49)	62.67 (52.32)	60.00 (50.75)	57.33 (49.20)
	E_2	46.67 (43.07)	65.33 (53.92)	70.67 (57.20)	74.67 (59.82)	76.00 (60.69)	82.67 (65.40)	90.67 (72.26)	58.67 (49.97)	50.67 (45.36)	44.00 (41.52)
	E_3	44.00 (41.54)	48.00 (43.84)	50.67 (45.36)	53.33 (46.89)	61.33 (51.54)	58.67 (49.97)	56.00 (48.43)	45.33 (42.30)	46.67 (43.07)	49.33 (44.60)
G_3	E_1	57.33	69.33	72.00	70.67	76.00	81.33	82.67	66.67	64.00	61.33

(No. 40)		(49.2)	(56.36)	(58.03)	(57.20)	(60.69)	(64.41)	(65.40)	(54.72)	(53.11)	(51.54)
	E_2	50.67 (45.36)	69.33 (56.36)	74.67 (59.77)	77.33 (61.56)	80.00 (63.48)	86.67 (68.60)	94.67 (76.80)	62.67 (52.32)	54.67 (47.66)	48.00 (43.83)
	E_3	49.33 (44.6)	53.33 (46.89)	56.00 (48.43)	58.67 (49.97)	66.67 (54.72)	62.67 (52.34)	60.00 (50.75)	50.67 (45.36)	50.67 (45.36)	54.67 (47.66)
G_4 (No. 24)	E_1	49.33 (44.6)	61.33 (51.54)	64.00 (53.11)	62.67 (52.32)	68.00 (55.56)	73.33 (58.90)	74.67 (59.77)	58.67 (49.97)	56.00 (48.43)	53.33 (46.89)
	E_2	42.67 (40.76)	61.33 (51.54)	66.67 (54.72)	70.67 (57.23)	72.00 (58.07)	78.67 (62.49)	86.67 (68.60)	54.67 (47.66)	46.67 (43.07)	40.00 (39.18)
	E_3	40.00 (39.22)	44.00 (41.54)	46.67 (43.07)	49.33 (44.60)	57.33 (49.2)	54.67 (47.66)	52.00 (46.13)	41.33 (39.99)	42.67 (40.76)	45.33 (42.30)
G_5 (No. 44)	E_1	61.33 (51.54)	73.33 (58.90)	76.00 (60.64)	74.67 (59.77)	80.00 (63.48)	84.00 (66.40)	86.67 (68.60)	70.67 (57.20)	68.00 (55.53)	65.33 (53.92)
	E_2	56.00 (48.43)	73.33 (58.90)	78.67 (62.49)	77.33 (61.56)	84.00 (66.5)	90.67 (72.26)	96.00 (78.43)	66.67 (54.72)	58.67 (49.97)	52.00 (46.14)
	E_3	53.33 (46.89)	57.33 (49.20)	60.00 (50.75)	62.67 (52.32)	69.33 (56.36)	66.67 (54.75)	64.00 (53.11)	54.67 (47.66)	54.67 (47.66)	58.67 (49.97)
G_6 (No. 28)	E_1	45.33 (42.3)	54.67 (47.66)	60.00 (50.75)	57.33 (49.20)	61.33 (51.54)	65.33 (53.92)	68.00 (55.53)	52.00 (46.13)	50.67 (45.36)	48.00 (43.84)
	E_2	37.33 (37.64)	53.33 (46.89)	58.67 (49.97)	62.67 (52.34)	64.00 (53.13)	70.67 (57.20)	78.67 (62.49)	46.67 (43.07)	38.67 (38.43)	32.00 (34.36)
	E_3	28.00 (31.94)	37.33 (37.64)	42.67 (40.76)	44.00 (41.54)	52.00 (46.13)	49.33 (44.60)	45.33 (42.30)	32.00 (34.44)	34.67 (36.05)	40.00 (39.22)
G_7 (No. 63)	E_1	41.33 (39.99)	50.67 (45.36)	56.00 (48.43)	53.33 (46.89)	57.33 (49.2)	61.33 (51.54)	64.00 (53.11)	48.00 (43.84)	46.67 (43.07)	44.00 (41.54)
	E_2	33.33 (35.24)	49.33 (44.60)	54.67 (47.66)	58.67 (49.99)	60.00 (50.76)	66.67 (54.72)	74.67 (59.77)	42.67 (40.76)	34.67 (36.05)	28.00 (31.83)
	E_3	24.00 (29.32)	33.33 (35.24)	38.67 (38.43)	40.00 (39.22)	48.00 (43.83)	45.33 (42.30)	41.33 (39.99)	28.00 (31.94)	30.67 (33.6)	36.00 (36.86)
G_8 (No. 37)	E_1	37.33 (37.64)	46.67 (43.07)	52.00 (46.13)	49.33 (44.60)	53.33 (46.89)	57.33 (49.20)	60.00 (50.75)	44.00 (41.54)	42.67 (40.76)	40.00 (39.22)
	E_2	29.33 (32.77)	45.33 (42.30)	50.67 (45.36)	54.67 (47.67)	56.00 (48.43)	62.67 (52.32)	70.67 (57.20)	38.67 (38.43)	30.67 (33.6)	24.00 (29.18)
	E_3	22.67 (28.4)	29.33 (32.77)	33.33 (35.24)	36.00 (36.86)	42.67 (40.76)	40.00 (39.20)	38.67 (38.43)	26.67 (31.06)	28.00 (31.94)	32.00 (34.44)
G_9 (No. 66)	E_1	33.33 (35.24)	42.67 (40.76)	48.00 (43.84)	45.33 (42.30)	49.33 (44.6)	53.33 (46.89)	56.00 (48.43)	40.00 (39.22)	38.67 (38.43)	36.00 (36.86)
	E_2	25.33 (30.19)	41.33 (39.99)	46.67 (43.07)	50.67 (45.37)	52.00 (46.13)	58.67 (49.97)	66.67 (54.72)	34.67 (36.05)	26.67 (31.06)	20.00 (26.36)
	E_3	16.00 (23.57)	25.33 (30.19)	30.67 (33.60)	32.00 (34.44)	40.00 (39.20)	37.33 (37.64)	33.33 (35.24)	20.00 (26.55)	22.67 (28.4)	28.00 (31.94)
G_{10} (Konkan Bhushan)	E_1	70.67 (57.2)	78.67 (62.49)	82.67 (65.40)	81.33 (64.41)	85.33 (67.50)	86.67 (68.60)	88.00 (69.70)	77.33 (61.56)	73.33 (58.9)	73.33 (58.90)
	E_2	74.67 (59.82)	85.33 (67.50)	89.33 (70.98)	86.67 (68.60)	92.00 (73.89)	93.33 (75.17)	96.00 (78.43)	81.33 (64.41)	78.67 (62.49)	77.33 (61.56)
	E_3	54.67 (47.66)	57.33 (49.20)	62.67 (52.32)	65.33 (53.92)	72.00 (58.03)	70.67 (57.20)	69.33 (56.36)	58.67 (49.97)	60.00 (50.76)	64.00 (53.13)
Range	min	16.00 (23.57)	25.33 (30.19)	30.67 (33.60)	32.00 (34.44)	40.00 (39.2)	37.33 (37.64)	33.33 (35.24)	20.00 (26.55)	22.67 (28.4)	20.00 (26.36)
	max	74.67 (59.82)	85.33 (67.50)	89.33 (70.98)	86.67 (68.60)	92.00 (73.89)	93.33 (75.17)	96.00 (78.43)	81.33 (64.41)	78.67 (62.49)	77.33 (61.56)

(Figures in parentheses indicate arcsine transformed value)

Table 6: Effect of Genotype, Explant and Medium on multiple shoot induction in *in vitro* regeneration

Genotypes		**SIM_1**	**SIM_2**	**SIM_3**	**SIM_4**	**SIM_5**	**SIM_6**	**SIM_7**	**SIM_8**	**SIM_9**	**SIM_{10}**
G_1 (No. 47)	E_1	0.00	3.00	4.33	3.67	4.67	6.33	6.67	2.33	2.67	2.33
	E_2	0.00	5.00	6.33	5.67	7.00	8.33	8.67	4.33	3.00	2.33
	E_3	0.00	3.67	5.67	4.67	6.67	6.33	7.00	4.00	3.00	2.00
G_2 (No. 15)	E_1	0.00	3.67	4.33	4.67	5.67	5.33	5.00	2.67	3.33	4.00
	E_2	0.00	5.00	6.33	5.67	7.00	8.33	8.67	4.33	3.00	2.33
	E_3	0.00	5.67	7.67	6.33	7.33	8.33	9.00	5.33	5.00	4.00
G_3 (No. 40)	E_1	0.00	5.67	6.33	6.67	7.67	7.33	7.00	4.67	5.33	6.00
	E_2	0.00	6.00	7.33	6.67	8.00	8.67	9.67	5.33	4.00	2.67
	E_3	0.00	6.67	8.67	7.33	8.33	9.33	10.00	6.33	6.00	5.00
G_4 (No. 24)	E_1	0.00	6.67	7.33	7.67	8.67	8.33	8.00	5.67	6.33	7.00
	E_2	0.00	4.00	5.33	4.67	6.00	7.33	7.67	3.33	2.33	2.00
	E_3	0.00	4.67	6.67	5.33	7.67	7.33	8.00	4.33	4.00	3.00

G_5 (No. 44)	E_1	0.00	7.00	8.33	7.67	9.00	9.67	10.67	6.33	5.00	3.67
	E_2	0.00	6.33	8.33	7.67	9.33	10.33	11.00	5.67	5.00	4.00
	E_3	0.00	7.67	8.33	8.67	9.67	9.33	9.00	6.67	7.33	8.00
G_6 (No. 28)	E_1	0.00	2.33	3.67	3.33	4.00	5.33	5.67	2.67	3.00	2.33
	E_2	0.00	2.67	4.67	3.67	5.67	5.33	6.00	2.33	2.67	2.00
	E_3	0.00	3.33	3.00	3.67	5.00	4.67	4.33	2.67	3.00	3.00
G_7 (No. 63)	E_1	0.00	4.00	4.33	4.00	4.67	5.00	5.33	3.67	3.67	3.33
	E_2	0.00	2.33	4.33	4.67	5.00	4.67	5.33	2.33	2.00	2.67
	E_3	0.00	3.00	3.67	4.00	4.67	4.33	4.00	2.33	2.67	3.33
G_8 (No. 37)	E_1	0.00	3.67	4.00	3.67	4.33	4.67	5.00	3.33	3.33	3.00
	E_2	0.00	2.00	3.33	3.67	4.00	3.67	5.67	2.67	2.33	2.33
	E_3	0.00	2.67	3.00	3.33	4.33	4.00	3.67	2.00	2.33	3.00
G_9 (No. 66)	E_1	0.00	3.00	3.67	3.33	4.00	4.33	4.67	2.67	2.67	2.33
	E_2	0.00	2.33	2.33	2.67	3.00	2.67	3.33	3.33	3.00	2.67
	E_3	0.00	2.00	2.33	2.67	3.67	3.33	3.00	1.67	2.00	2.33
G_{10} (Konkan Bhushan)	E_1	0.00	7.33	8.67	8.00	9.67	10.00	11.00	6.67	5.33	4.00
	E_2	0.00	6.67	8.67	8.00	9.67	10.67	11.33	6.00	5.67	4.67
	E_3	0.00	8.00	8.67	9.67	10.67	10.33	10.00	7.00	7.67	8.33
Range	min	0.00	2.00	2.33	2.67	3.00	2.67	3.00	1.67	2.00	2.00
	max	0.00	8.00	8.67	9.67	10.67	10.67	11.33	7.00	7.67	8.33

Factors	Genotype (G)	Explant (E)	Medium (M)	Interaction G × E × M
SE(m)	0.06	0.03	0.06	0.31
C.D. at 1 %	0.16	0.09	0.16	0.85
Significant at 1 %	Sig	Sig	Sig	Sig

Table 7: Effect of Genotype and Medium on days to shoot induction in *in vitro* regeneration

G/M		SIM1	SIM2	SIM3	SIM4	SIM5	SIM6	SIM7	SIM8	SIM9	SIM10	Mean A
G_1	No. 47	8.89	8.67	8.56	8.67	8.00	7.44	7.56	8.00	8.33	8.89	8.30
G_2	No. 15	8.78	7.78	6.89	7.44	6.89	6.33	6.11	8.22	8.67	8.78	7.59
G_3	No. 40	9.00	7.33	7.00	7.00	6.33	6.33	6.00	7.67	8.56	8.33	7.36
G_4	No. 24	8.44	8.22	7.67	7.56	7.33	7.00	6.78	8.44	7.78	8.78	7.80
G_5	No. 44	8.33	7.11	6.33	6.44	5.78	5.56	5.44	7.67	8.00	8.11	6.88
G_6	No. 28	9.22	9.00	8.89	9.00	8.33	8.33	7.78	8.11	8.56	9.33	8.66
G_7	No. 63	9.89	9.33	9.44	9.11	8.89	8.67	8.33	9.11	9.00	10.22	9.20
G_8	No. 37	10.00	9.78	9.56	9.89	9.11	9.11	8.56	9.11	9.44	10.22	9.48
G_9	No. 66	10.00	10.11	10.11	10.00	9.78	8.89	8.89	9.44	9.67	10.00	9.69
G_{10}	Konkan Bhushan	8.22	6.44	5.78	5.89	4.89	4.67	4.67	7.00	7.22	7.33	6.21
Range	Min	8.22	6.44	5.78	5.89	4.89	4.67	4.67	7.00	7.22	7.33	6.21
	Max	10.00	10.11	10.11	10.00	9.78	9.11	8.89	9.44	9.67	10.22	9.69
Mean (Explant)		9.08	8.38	8.02	8.10	7.53	7.23	7.01	8.28	8.52	9.00	

Factors	Genotype (G)	Medium (M)	Interaction G × M
SE(m)	0.071	0.071	0.223
CD. at 1 %	0.260	0.260	0.815
Significant at 1 %	Sig	Sig	Sig

Table 8: Effect of Genotype and Medium on shoot elongation frequency in *in vitro* regeneration

G/M		SIM_1	SIM_2	SIM_3	SIM_4	SIM_5	SIM_6	SIM_7	SIM_8	Mean
G_1	No. 47	0.00 (0.00)	55.56 (48.2)	46.67 (43.09)	31.11 (33.87)	24.44 (29.58)	40.00 (38.86)	48.89 (44.36)	42.22 (40.52)	36.11 (34.81)
G_2	No. 15	0.00 (0.00)	68.89 (56.13)	60.00 (50.77)	35.56 (36.59)	33.33 (35.26)	82.22 (65.15)	55.56 (48.20)	48.89 (44.36)	48.06 (42.06)
G_3	No. 40	0.00 (0.00)	75.56 (60.42)	66.67 (54.74)	44.44 (41.80)	33.33 (35.26)	88.89 (70.73)	62.22 (52.09)	55.56 (48.20)	53.33 (45.41)
G_4	No. 24	0.00 (0.00)	62.22 (52.09)	53.33 (46.91)	37.78 (37.91)	26.67 (31.09)	82.22 (65.15)	55.56 (48.20)	48.89 (44.36)	45.83 (40.71)
G_5	No. 44	0.00 (0.00)	80.00 (63.43)	73.33 (58.91)	51.11 (45.64)	40.00 (39.23)	93.33 (75.04)	68.89 (56.13)	62.22 (52.09)	58.61 (48.81)
G_6	No. 28	0.00 (0.00)	48.89 (44.36)	40.00 (39.23)	24.44 (29.58)	22.22 (28.07)	68.89 (56.13)	42.22 (40.52)	35.56 (36.59)	35.28 (34.31)
G_7	No. 63	0.00 (0.00)	42.22 (40.52)	33.33 (35.26)	22.22 (28.07)	24.44 (29.58)	62.22 (52.09)	35.56 (36.59)	28.89 (32.48)	31.11 (31.82)
G_8	No. 37	0.00 (0.00)	31.11 (33.87)	26.67 (31.09)	15.56 (23.13)	17.78 (24.85)	55.56 (48.20)	28.89 (32.48)	22.22 (28.07)	24.72 (27.71)
G_9	No. 66	0.00 (0.00)	24.44 (29.58)	20.00 (26.57)	13.33 (21.42)	17.78 (24.85)	48.89 (44.36)	22.22 (28.07)	15.56 (23.13)	20.28 (24.75)

G_{10}	Konkan Bhushan	0.00 (0.00)	84.44 (66.87)	82.22 (65.15)	68.89 (56.13)	62.22 (52.09)	100.00 (90.00)	75.56 (60.42)	71.11 (57.52)	68.06 (56.02)
Range	Min	0.00 (0.00)	24.44 (29.58)	20.00 (26.57)	13.33 (21.42)	17.78 (24.85)	40.00 (38.86)	22.22 (28.07)	15.56 (23.13)	20.28 (24.75)
	Max	0.00 (0.00)	84.44 (66.87)	82.22 (65.15)	68.89 (56.13)	62.22 (52.09)	100.00 (90.00)	75.56 (60.42)	71.11 (57.52)	68.06 (56.02)
Mean		0.00 (0.00)	57.33 (49.55)	50.22 (45.17)	34.44 (35.41)	30.22 (32.99)	72.22 (60.57)	49.56 (44.71)	43.11 (40.73)	

(Figures in parentheses indicate arcsine transformed value)

	Genotype	**Medium**	**Genotype × Medium**
SEm	0.917	0.820	2.594
CD at 1 %	3.381	3.024	9.563
Significant at 1 %	Sig	Sig	Sig

Rooting and establishment

As the survivability of plantlets depends mainly on number and type of roots produced from shoots, it was necessary to find out proper auxins and its concentration for inducing roots. Auxins such as IAA, IBA and NAA are most commonly used to induce rooting both in *in vitro* and *ex vitro* conditions. Earlier researchers were not successful to establish the complete plantlets [12]. However, complete establishment of plant done by Geetha *et al*. [7] with 90 to 95 per cent frequency. *In vitro* formed roots are obtained which were thin and lack roots hairs. These roots are frequently died or collapsed after the plantlets are removed from culture. There is a need to develop efficient rooting and establishment system, so that the recovery of maximum transgenic plants will possible.

In the present study, highest root induction frequency (79.51%) was observed on medium MS+ 1.0 mg/l IBA+ 2% sucrose (Table 10). Comparing capability of IBA, NAA, BAP, maximum number of roots (7.67) were developed in media combination MS + 1 mg/l NAA + 2% sucrose and MS + 0.5 mg/l NAA (Table 11). Similar result were observed by Yadav and Chand1 [3], Naz *et al*. [8] and Sarode [14]. They reported that NAA promoted better root formation as compared to IBA and it indicated that frequency of functional rooting increased with strength of medium. The genotype Konkan Bhushan showed early root induction (8.33 days) as compared to other genotypes (Table 9).

Table 9: Effect of Genotype and Medium on days to root induction in *in vitro* regeneration

G/M		**RIM_1**	**RIM_2**	**RIM_3**	**RIM_4**	**RIM_5**	**RIM_6**	**RIM_7**	**RIM_8**	**RIM_9**	**Mean**
G_1	No. 47	0.00	17.00	16.33	16.00	14.00	12.00	15.33	12.67	16.67	13.33
G_2	No. 15	0.00	16.00	15.00	13.67	12.00	13.33	11.33	15.67	15.33	12.48
G_3	No. 40	0.00	15.00	14.00	12.67	11.33	12.33	10.33	14.67	14.33	11.63
G_4	No. 24	0.00	16.67	15.67	15.00	13.00	14.33	11.67	16.33	15.33	13.11
G_5	No. 44	0.00	14.00	13.00	12.33	10.33	11.33	9.33	13.67	13.33	10.82
G_6	No. 28	0.00	16.00	15.67	15.00	14.00	12.33	13.67	13.33	16.33	12.93
G_7	No. 63	0.00	16.33	15.33	16.00	15.00	12.33	12.67	13.33	15.67	12.96
G_8	No. 37	0.00	15.67	15.33	17.00	16.00	10.67	12.67	12.33	15.33	12.78
G_9	No. 66	0.00	15.67	15.00	16.33	15.33	10.67	12.33	12.67	14.67	12.52
G_{10}	Konkan Bhushan	0.00	12.33	10.67	10.33	9.33	9.67	8.33	11.67	11.33	9.30
Range	Min	0.00	12.33	10.67	10.33	9.33	9.67	8.33	11.67	11.33	9.30
	Max	0.00	17.00	16.33	17.00	16.00	14.33	15.33	16.33	16.67	13.33
Mean		0.00	15.47	14.60	14.43	13.03	11.90	11.77	13.63	14.83	

	Genotype	**Medium**	**Genotype × Medium**
SEm	0.101	0.096	0.302
CD at 1 %	0.369	0.351	1.104
Significant at 1 %	Sig	Sig	Sig

Table 10: Effect of Genotype and Medium on root induction frequency in *in vitro* regeneration

G/M		**RIM_1**	**RIM_2**	**RIM_3**	**RIM_4**	**RIM_5**	**RIM_6**	**RIM_7**	**RIM_8**	**RIM_9**	**Mean**
G_1	No. 47	0.00(0.00)	20.00 (26.36)	48.89 (44.35)	57.78 (49.52)	62.22 (52.09)	60.00 (50.77)	68.89 (56.13)	31.11 (33.87)	46.67 (43.09)	43.95 (39.58)
G_2	No. 15	0.00 (0.00)	33.33 (35.2)	62.22 (52.13)	68.89 (56.13)	77.78 (61.93)	73.33 (58.91)	82.22 (65.15)	44.45 (41.8)	60.00 (50.77)	55.80 (46.89)
G_3	No. 40	0.00 (0.00)	40.00 (39.19)	68.89 (56.2)	75.55 (60.42)	84.45 (66.87)	80.00 (63.43)	88.89 (70.73)	51.11 (45.64)	66.67 (54.74)	61.73 (50.8)
G_4	No. 24	0.00 (0.00)	26.67 (30.97)	55.56 (48.21)	64.44 (53.48)	71.11 (57.52)	66.67 (54.74)	75.55 (60.42)	37.78 (37.91)	53.33 (46.91)	50.12 (43.35)
G_5	No. 44	0.00 (0.00)	46.67 (43.08)	75.56 (60.54)	82.22 (65.15)	91.11 (72.89)	86.67 (68.58)	93.33 (75.04)	57.78 (49.48)	73.33 (58.91)	67.41 (54.85)
G_6	No. 28	0.00 (0.00)	22.22 (28.07)	48.89 (44.35)	57.78 (49.52)	64.45 (53.41)	60.00 (50.77)	66.67 (54.74)	31.11 (33.87)	46.67 (43.09)	44.20 (39.76)
G_7	No. 63	0.00	15.55	13.33	17.78	20.00	20.00	20.00	15.55	22.22	16.05

		(0.00)	(23.13)	(21.42)	(24.85)	(26.36)	(26.57)	(26.36)	(23.13)	(28.07)	(22.21)
G_8	No. 37	0.00 (0.00)	17.78 (24.85)	13.33 (21.42)	15.55 (23.13)	17.78 (24.85)	15.55 (23.13)	20.00 (26.36)	24.45 (29.58)	22.22 (28.07)	16.30 (22.38)
G_9	No. 66	0.00 (0.00)	20.00 (26.36)	20.00 (26.57)	15.55 (23.13)	13.33 (21.42)	17.78 (24.85)	22.22 (28.07)	24.45 (29.58)	20.00 (26.36)	17.04 (22.93)
G_{10}	Konkan Bhushan	0.00 (0.00)	75.55 (74.34)	88.89 (69.4)	91.11 (71.11)	97.78 (72.62)	95.55 (74.55)	100.00 (80.79)	82.22 (82.3)	84.45 (82.5)	79.51 (67.51)
Range	Min	0.00 (0.00)	15.55 (23.13)	13.33 (21.42)	15.55 (23.13)	13.33 (21.42)	15.55 (23.13)	20.00 (26.36)	15.55 (23.13)	20.00 (26.36)	16.05 (22.21)
	Max	0.00 (0.00)	75.55 (74.34)	88.89 (69.4)	91.11 (71.11)	97.78 (72.89)	95.55 (74.55)	100.00 (80.79)	82.22 (82.3)	84.45 (82.5)	79.51 (67.51)
Mean		0.00 (0.00)	31.78 (30.8)	49.56 (41.69)	54.67 (45.04)	60.00 (48.59)	57.56 (46.86)	63.78 (51.44)	40.00 (36.1)	49.56 (42.22)	

(Figures in parentheses indicate arcsine transformed value)

	Genotype	**Medium**	**Genotype × Medium**
SEm	0.808	0.766	2.423
CD at 1 %	2.954	2.800	8.858
Significant at 1 %	Sig	Sig	Sig

Table 11: Effect of Genotype and Medium on number of roots per shoot in *in vitro* regeneration

G/M		**RIM_1**	**RIM_2**	**RIM_3**	**RIM_4**	**RIM_5**	**RIM_6**	**RIM_7**	**RIM_8**	**RIM_9**	**Mean**
G_1	No. 47	0.00	2.67	2.33	3.67	4.67	4.00	3.67	4.00	2.67	3.07
G_2	No. 15	0.00	2.00	3.00	3.33	4.33	4.00	5.00	2.33	2.67	2.96
G_3	No. 40	0.00	3.00	4.00	4.33	5.33	5.00	6.00	3.33	3.67	3.85
G_4	No. 24	0.00	2.33	2.67	3.33	4.67	5.00	4.33	2.67	2.33	3.04
G_5	No. 44	0.00	4.00	5.00	5.33	6.33	6.00	7.00	4.33	4.67	4.74
G_6	No. 28	0.00	2.33	3.00	2.33	3.67	4.00	3.33	2.67	2.00	2.59
G_7	No. 63	0.00	2.33	2.00	2.67	3.00	3.00	3.00	2.33	3.33	2.41
G_8	No. 37	0.00	2.67	2.00	2.33	2.67	2.33	3.00	3.67	3.33	2.44
G_9	No. 66	0.00	3.00	3.00	2.33	2.00	2.67	3.33	3.67	3.00	2.56
G_{10}	Konkan Bhushan	0.00	4.00	5.33	5.67	7.33	6.33	7.67	4.33	4.67	5.04
Range	Min	0.00	2.00	2.00	2.33	2.00	2.33	3.00	2.33	2.00	2.41
	Max	0.00	4.00	5.33	5.67	7.33	6.33	7.67	4.33	4.67	5.04
Mean		0.00	2.83	3.23	3.53	4.40	4.23	4.63	3.33	3.23	

	Genotype	**Medium**	**Genotype × Medium**
SEm	0.099	0.094	0.298
CD at 1 %	0.362	0.344	1.089
Significant at 1 %	Sig	Sig	Sig

Gradual acclimatization is necessary to these plants to survive transition from culture to the green house. Inspite of considerable tissue culture protocol for successful regeneration of complete plantlets, the establishment of plants in soil is difficult and very few reports have discussed the problems involved in plant establishment. The difficulties were experienced by some workers to resort inefficient *in vitro* grafting by utilizing germinated seedlings and rootstocks [15]. However, such methods are not only time consuming but technique specific and success rates vary among different hands.

In the present study, establishment of plantlets was found very easy by manipulation of potting mixture. The equal quantity of soil, sand and vermiculite provide compactness, aeration and nutrient which results into 73.33 per cent survival rates (Table 12). This may be due to effect of growth chambers treatment where humidity maintained at 95% around the plant to which they got adapted during culture. The humidity was gradually reduced to the ambient levels over a period of 2 to 4 weeks. This probably re-commissions the photosynthetic machinery of the plants, enabling them to withstand the subsequent reduction in the ambient relative humidity and survival under green house. While in only soil or sand the plant mortality was more. Similar hardening procedure was adopted in pigeon pea and other pulses by Geetha *et al.* [7], Rathore and Chand [16] and Sarode [14].

Table 12: Hardening and establishment of *in vitro* shoots in different potting mixture

Treatment			**Number of plants shifted**	**Number of plants survived**	**Survivability (%)**
Soil	**Vermiculate**	**Sand**			
1.00	0.00	0.00	30	12	40.00
1.00	1.00	0.00	30	13	43.3
1.00	1.00	1.00	30	22	73.33
1.00	0.00	1.00	30	10	33.33

Conclusion

From present piece of work, it can be concluded that the *in vitro* regenerability is dependent on genotypic characters as well as growth regulator combinations. The genotype Konkan Bhushan followed by genotype G_9 (No. 66) were proved better for regeneration and could be further taken for genetic

manipulation studies. An efficient regeneration technique for a dolichos bean Cv. Konkan Bhushan could be put forth from the study.

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