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A Study on the Response of Three Genotypes of *Mucuna pruriens* to Different Growth Media for Tissue Culture Differentiation

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Abbreviations

6-Benzylaminopurine	BAP
Citric acid	CA
Casein Hydrolysate	CH
Gibberellic acid	GA_3
Gamborgs B-5 basal media	G-B5
Indol-3-Acetic Acid	IAA
Indol-3-Butyric Acid	IBA
2-isopentenyladenine	2-iP
Kinetin	Kn
Murashige and Skoog basal media	MS
α-Naphthaleneacetic acid	NAA
Revised Tobacco medium	RT
Thidiazuron [1-Phenyl-3- (1,2,3-Thiadiazol-5-yl) Urea]	TDZ

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Differential Response of Different Growth Media for Tissue Culture Differentiation in Three Genotypes of *Mucuna Pruriens*

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Abstract

In vitro regeneration using meristem explants to induce shoots and roots in *Mucuna pruriens* genotypes were investigated. Different treatment combinations of culture media containing growth hormones, nutrients and vitamins were evaluated to promote shoots and roots directly from the meristem explants and indirectly via callus phase. A total of 13 experiments were conducted. Among the various cytokinins, BAP, Kn, 2iP, GA3 and TDZ tested, the survival of tissue (shoots and callus) was found to be effective in BAP combined with an auxin NAA. The cytokinins, BAP and TDZ induced multiple shoots. An optimal level of 0.1mg/l BAP supplemented with liquid media containing ¼ G-B5 + 7.5 g/l sucrose supplemented with 5mg/l IBA was effective in root induction. BAP along with an auxin NAA was effective for survival of tissues (shoots as well as callus). Rooting was induced at the base of shoots proliferated on media containing lower concentrations of BAP. However, shoots proliferated in TDZ containing media were difficult to root. No organogenesis was observed from callus regardless of the different media evaluated. Among the accessions, 10084, 14880 and 15169, accession 14880 performed relatively better in tissue survival percentage as well as percent of shoot survival. Results from callus and shoots were often different and should not be compared.

Keywords: Fabaceae, *Mucuna pruriens*, meristem culture, tissue culture

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1. INTRODUCTION

Mucuna pruriens belongs to the family Fabaceae (Leguminosae), which are a large group of flowering plants with a world-wide distribution. Many are economically or ecologically important. The family consists of about 18,000 species in 630 genera (Mohan and Krishnamurthy, 2003). Legumes are the most important crop plants next to cereals. They are known for their high protein content, lysine and biological nitrogen fixation because of symbiotic association with microorganisms that fix atmospheric nitrogen. They can serve as vitamin rich food (grain legumes) and feed (forage and pasture legumes), as well as ornamental plants. Some also have high value timber and important medicinal uses.

The genus *Mucuna* has been reported to contain about 100 species of both wild and domesticated found in tropical and subtropics. Among the species in this genus *Mucuna pruriens* is the one that is widely cultivated and well investigated (Lorenzetti, *et. al.,* 1998). *Mucuna* species are climbing, fast growing annual plants with white to dark purple flowers, susceptible to waterlogging and relatively tolerant to drought and most soil types, i.e. soils with sandy to sandy-clay texture, pH of 5.0-7.0 and low fertility. *Mucuna* performs best in a broad range of relatively high rainfall (1000-2500mm annual rainfall) and elevation (0-1600 m above sea level) areas. It tolerates a relatively narrow range of temperatures (19-27°C) but it is still adaptable to humid and semi-humid zones of West Africa (FAO, 1982). *Mucuna* grows better in areas with a bi-modal rainfall regime. It produces 7-9 t/ha of dry matter in areas with bi-modal rainfall and in areas with lesser rainfall (700-900 mm) the foliage yield can be reduced by half. The seed yield is about 1.3-2 t/ha. The life cycle of *Mucuna* varies between 100 and 290 days (Buckles *et al.*, 1998). It is an indigenous crop of tropical regions, especially Africa, India, and the West Indies. It can be used either as a fodder crop, a green manure as well as a cover crop. Since *Mucuna* fixes nitrogen, it is cultivated as a fallow crop to improve soil fertility, as a smoother crop to control weeds and also as a forage plant. *Mucuna* is also well known for its nematicidal effect in rotation cropping with other commercial crops although it is not itself immune to a number of nematode species. Moreover, it increases the yield of cereals, especially maize when intercropped with it, specifically if the biomass is left on the soil and incorporated or in rotation cropping by

increasing the yield of subsequent crop. It better fits in rotation cropping than intercropping due to its climbing type (Duke, 1981; Vissoh *et al.,* 2003).

The seeds of *Mucuna* are high in proteins (23-35%), carbohydrates (65%), lipids, fiber, and minerals (Bressani, 2002). *Mucuna* species have been roasted and grounded to make a coffee substitute for several decades. It is widely known as -Nescafé for this reason. The bean and/or pods are cooked as vegetables by the ketchi natives in Guatemala (Buckles *et al.*, 1998). Besides, *Mucuna* seeds are important in the management of Parkinson's disease by virtue of their L-DOPA (4-3, 4-dihydroxy phenylalanine) content.

Such multiple purpose crops have the potential to sustain agriculture and ensure food security for smallholder farmers by maintaining soil fertility and serve as both feed or food and forage. The fact that smallholder farmers have limited financial resources to purchase artificial fertilizers; the ability of the crop to easily establish and endure hard conditions; and financial advantage of organically grown produces, to mean using the crop as green manure encourages adopting this crop. However, need for a ready cash return, seed availability, loss of the grains due to biomass incorporation for green manure and toxicity problems may still restrict its adoption by smallholder farmers (Maasdeorp, 2002).

The ILRI (International Livestock Research Institute) based at Addis Ababa has a large germplasm collection of forages. All the accessions of *Mucuna pruriens*, however, are infected with seed borne (transmitted) virus diseases. Preliminary tests using ELISA techniques were done in the laboratory and detected three major viruses, namely: bean common mosaic virus (BCMV), cowpea mosaic virus (CPMV) and cowpea severe mosaic virus (CPSMV). Eradication of virus pathogens of diseased plants is highly desirable in order to maximize their yield and also to facilitate the movement of materials across international boundaries. We know that there is no commercially available treatment to cure virus-infected plants. The therapeutic chemicals capable of eradicating virus from infected plants are either not available or considered environment unfriendly. Apical tips of shoot and root (meristems) are generally free from viruses or carry a very low concentration of the viruses. Ball (1946) demonstrated the possibility of regenerating plants *in vitro* from the shoot apices of certain angiosperms, which became an important tool for propagation of disease (virus)-free plant

such as virus free dahlias (Morel and Martin, 1952); citrus, potato and cassava (Walkey, 1980); and sugarcane (Hendre *et al.,* 1975, cited by Iyer, 1997). The escape of meristems from virus invasion is due to the absence of vascular system through which viruses move readily within the plant body. A high metabolic activity occurs in the meristematic cells, which do not allow virus replication. A high endogenous auxin level in shoot apices also inhibits virus multiplication. Although *in vitro* culture of meristems, commonly known as meristem culture, combined with heat treatment of the mother plant is a standard procedure for virus elimination, regeneration of shoots and roots from the meristem tissues is often difficult and time consuming. Several attempts have been made in the past regarding the development of a suitable protocol for *in vitro* shoot and root regeneration of plants in general and legumes in particular. Successful regeneration of legumes has been aided by species-specific determination of critical regeneration parameters such as explants source, genotype, media constituents and temperature (Khawar and Ozcan, 2002). Meristem culture is mostly applied in horticulture for virus elimination, though reports also exist in several field crops like *Trifolium* (Farid *et. al.*, 2004) and many others. According to Juan *et al* (2004), L2 (Phillips and Collins, 1979) medium supplemented with 0.003 mg/l of 4-amino-3, 5, 6-trichloropicolinic acid (PIC) and 1.0 mg/l of BAP gave consistent better results to propagate, maintain and eliminate viruses from elite red clover clones. Effective and reproducible regeneration of chickpea has been possible using explants derived from axillary meristems from the cotyledonary node of seedling explants in the culture media containing MS with low concentration of TDZ, 2iP and Kn (Jayanand *et al.,* 2003). *In-vitro* plant regeneration is possible either directly by induction of shoot morphogenesis and organogenesis or through an intervening callus phase. It is possible to induce organogenesis, when using proper explants and optimizing culture conditions. Few attempts have been made in rapid *in-vitro* propagation of *Mucuna pruriens* using seedling explants (Chattopadhyay *et al.,* 1995) and cell suspension culture or production of secondary metabolites for pharmaceuticals (L-DOPA) (Chattopadhyay *et al.,* 1994).

The first reports of using meristem culture to eliminate viruses in *Mucuna pruriens* are from ILRI. However, difficulties were encountered to successfully regenerate and proliferate further shoots from these meristems (A. Jorge, personal communication, 2004). These difficulties led to the beginning of this work that aims to induce shoot proliferation in order

to produce large number of *in vitro* plants that could be successfully acclimatized in green houses. These would further produce virus free seeds to replace the currently infected materials in the ILRI forage gene bank.

1.1. Objectives

The present study was carried out to investigate the differential responses of 3 different genotypes of *Mucuna pruriens* to different treatment combinations of culture media (hormones, nutrients and vitamins) on the regeneration of plants from meristem explants.

The specific objectives are:

To investigate the differential responses of treatment combinations in tissue culture growth using three major approaches:

- To promote shoots in tissue cultured callus
- To proliferate and elongate tissue cultured shoots
- To promote roots in tissue cultured shoots

2. LITERATURE REVIEW

2.1. *In vitro* propagation

In vitro plant tissue culture involves regeneration of entire plants from pieces of plant tissues or explants because of totipotency, i.e. capability to give rise to new identical plants. It has various applications in plant improvement and disease eradication. The success of plant tissue culture depends on the type of plant material, i.e. dicotyledons regenerate better than monocotyledons, and gymnosperms have very limited regenerative capacity except when juvenile. There are even differences within single species where age, type and size of explants can influence plant responses. Nutritional media, environment and plant growth regulators also affect the success of plant tissue culture (Razdan, 2002).

The first major achievements in plant tissue culture were reported on potato (Nobecourt, 1939), tobacco (White, 1939) and carrot (Gautheret, 1939). These first attempts were on the production of callus, which is unorganized masses of tissue, from cambial tissue (cited by Rawal, 1997; Gamborg, 2002). Later on, organogenesis and the production of whole functional plants were possible.

2.2. Legume tissue culture

Legumes are notoriously recalcitrant to tissue culture. The most prevalent mode of regeneration in grain legumes has been reported *via* direct organogenesis from cotyledonary node explants. Shoot apices, leaflets and embryo axes were also common regeneration

pathways for direct organogenesis. Several reports indicated that most of the explants are responsive to cytokinins, especially BAP and TDZ. Regeneration *via* callus has been poor in many legumes though some legumes like soybean and pea shoots were recovered from callus tissue at a low frequency (Atika and Deepak, 2003).
Multiple shoot regeneration of *Mucuna pruriens* was possible through a callus stage using seedling explant cultured on Revised Tobacco (RT) medium supplemented with 2.7 μM NAA and 9.8 μM 2iP. They were then rooted in half-strength liquid RT medium supplemented with 2.7 μM NAA (Chattopadhyay *et al.,* 1995).

In peas *(Pisum sativum)*, individual shoot development was induced from meristems using half strength Gamborg-B5 (Gamborg, *et al.,* 1968) medium supplemented with 0.2 mg/l of NAA. MS medium supplemented with B5 vitamins + BAP + NAA also induced multiple shoot regeneration from both shoot apices and lateral buds of peas. Shoots were also rooted later on the same medium containing 0.93 mg/l of NAA and no BAP (Kartha *et al.,* 1974). Malik and Saxena (2004) have reported that TDZ had a better response than Kn or Zeatin when using seed explant of peas. A wide concentration (5-50 μM) of TDZ induced a high frequency of shoots after 3 to 4 weeks. Rooting of these shoots was possible on modified MS + 2.5 μM NAA.

There are various reports of *in vitro* shoot formation in lentils (*Lens culinaris*) but with limited success in whole plant regeneration. Polanco *et al*. (1988) suggested the formation of multiple shoots on Murashige and Skoog (1962) medium with 2.0 mg/l of BAP and 0.2 mg/l of NAA. Moreover, Khanam (1994) and Khanam *et al.,* (1995) reported best result in multiple shoot regeneration on MS medium containing 0.5 mg/l of BAP + 0.5 mg/l of kinetin + 0.2 mg/l of NAA + 100 mg/l of CH. On the other hand, Sarker *et al.* (2003) obtained healthy shoots with well developed leaves on MS medium supplemented with 0.5 mg/l of BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 and 5.5 mg/l tyrosine, though various concentrations and combinations of these growth hormones initiated shoot formation. Khalid *et al.* (2004) reported rapid and simple shoot regeneration from cotyledonary nodes and stem nodes on MS + TDZ. Stem nodes produce shoot only in 0.25 and 0.5 mg/l of TDZ while cotyledonary nodes were produced in all the four concentrations tested (0.25, 0.5, 1.0, and 2.0). However, higher TDZ concentration reduced shoot regeneration and resulted in stunted shoots. They

also found TDZ to inhibit rooting due to the high cytokinin activity and "carry-over" effect. Root induction at the base of the *in vitro* regenerated shoots is still a serious problem. Previous reports indicated that root induction in lentils was achieved on a medium containing NAA or IAA (Polanco *et al.*, 1988) and also on half strength BS medium with no supplement of hormones (Warkentin and McHughen 1993). High concentration of IBA (1000 mg/l) followed by subculturing into 10.0 mg/l of IBA containing half MS medium induced rooting in lentils (Khanam *et al*., 1995). For long period of time there had been no report on rooting except that few roots appear sporadically at the base of regenerated shoots. However, Khawar and Ozcan (2002) have shown that 0.25 mg/l of IBA resulted in 25% rooting along with an average of 7.87 roots per shoot with a mean length of 7.13 mm all arising only after an initial callusing stage. Polanco and Rulz (1997) also suggested that BAP has an inhibitory effect on rooting in lentils.

Most of the studies reported for chickpea (*Cicer arietinum* L.) regeneration have been possible based on cotyledonary nodes or shoot apices derived from seedling explants. Pawan *et. al.* (2001) reported the lack of reproducibility of regeneration protocols and highly problematic rooting and subsequent acclimatization of the *in vitro* regenerated shoots. Using cotyledon explants, callus induction was noticed in all media formulation tested using either MS or B5 nutrients plus several concentrations of auxins (2,4-D, NAA, IAA) and cytokinins (BAP, Kn) but a wide variation existed in the percentage and weight of callus. Highest (95%) callus induction and maximum number of shoot buds were observed in MS + 3.0 mg/l 2,4-D + 3.0 mg/l BAP. Whereas, highest callus weight (0.701 g) was observed in B5 + 3.0 mg/l 2,4-D + 1.0 mg/l BAP. Highest (40%) shoot regeneration was possible on MS + 2.0 mg/l BAP + 0.5 mg/l NAA producing 2.50 shoots per callus followed by (32%) in 2.0 mg/l Kn + 0.5 mg/l IAA, where the number of shoots per callus was 3.33. Callus produced shoots only when cytokinins (BAP and Kn) were combined with auxins (NAA and IAA) (Huda *et al.,* 2003). Explants of shoot primordium of mature embryo were used for high percent regeneration. Callus induction was observed on MS + 0.1 mg/l NAA + 0.5 mg/l 2,4-D and plants regenerated on MS + 0.5 mg/l Kn + 0.2 mg/l BAP + 0.2 mg/l IAA (Altinkut *et al.*, 2003). According to Jayanand *et al.* (2003), it is also possible to regenerate whole plants using axillary meristem explants. A low level of TDZ (4 µM) was then most effective while levels of TDZ up to 10 µM resulted in better number of shoots but higher concentration of

TDZ resulted in stunted growth. TDZ (4 µM) in combination with 2-iP (10 µM) + Kn (2 µM) showed best results both at the end of 2 weeks (23 shoots) and 4 weeks (40 shoots). Prolonged culture of explants on TDZ containing media reduced the numbers of induced multiple shoots. Transfer of induced shoots to shoot elongation media containing mainly 2-iP, Kn and GA_3 resulted in elongation of stunted shoots. 2,4-D alone promoted root formation (MS / B5 + 1 and 3 mg/l 2,4-D). However, ½ MS + 1.0 mg/l IBA were most effective for rooting of shoots in chickpea.

In sanfoin (*Onobrychis viciifolia*), a forage legume, highest shoot multiplication was recommended on MS medium supplemented with 2 mg/l of BAP plus 0.05 or 0.1 mg/l of IBA. Also, 2 mg/l of BAP plus 0.05, 0.1 or 0.5 mg/l of NAA and 8 mg/l of BAP plus 0.05 mg/l of NAA were recommended. Generally, high BAP level was not recommended since it decreased the number of shoots as well as the shoot length. Rooting was optimum at 1 mg/l (less concentration) of IBA supplemented to MS medium (Sancak, 1999).

In cowpea (*Vigna unguiculata*), shoot regeneration was possible on 1/3 MS medium supplemented first with BAP (15-35) for initiation followed by 1.0 mg/l of BAP. But the regeneration efficiency varies among different genotypes. On the other hand, 1.0 mg/l of IAA or 0.05 mg/l of NAA were found to promote rooting though hormone-free MS medium was also sufficient (Machuka *et. al,* 2000).

According to Ochatt *et al.* (2001), the optimum hormonal combination for regeneration of grasspea *(Lathyrus sativus)* varieties was genotype specific. Some genotypes of grasspea responded best on auxin- free medium with 5.0 mg/l of BAP, while others performed better with 0.01 mg/l of NAA + 5.0 mg/l of BAP or even only on 3.0 mg/l of BAP. Rooting was optimum on half strength hormone-free MS medium, though differences appeared within genotypes.

3. MATERIALS AND METHODS

3.1. General procedures

A series of 13 experiments were carried out over a period of 9 months between November and July 2005 at the Tissue Culture Laboratory, Forage Diversity Unit, ILRI, Addis Ababa. A protocol was developed for each experiment and the various media tested were labeled with a letter and sequential numbers, following the existent routine at the Laboratory. Media developed earlier or parallel to ILRI's experiments were labeled with "M", while media developed for the student's experiments were labeled with "H" (Table 1).

Table 1. Media List

Media Code	**Sucrose**	**G – B5**	**MS** (g/l)	**MS***	**Agar** (g/l)	**BAP** (mg/l	**2-iP** (mg/l)	**Kn** (mg	***NAA***	**TDZ** (mg/l	**Ad.** (mg/l	**Th.** (mg/l)	**CA** (mg/l)	**GA** (mg/	**RT** (m	**Inos Itol***	**Vit. B12**

	(g/l)	(g/l)		(g/l)		)		/l)	(mg/l)	)	)			l)	g/l)	(g/l)	(mg/l)
M29	30	3.2	-	-	8	0.5	-	-	0.1	-	-	-	100	0.3	-	-	-
M35	30	3.2	-	-	8	0.5	-	-	0.5	-	-	-	100	0.3	-	-	-
M32/ H1	30	3.2	-	-	8	0.1	-	-	-	-	2	20	100	-	-	-	-
H2	30	3.2	-	-	8	-	-	0.5	-	-	2	20	100	-	-	-	-
H3	30	3.2	-	-	8	-	-	1	-	-	2	20	100	-	-	-	-
H4	30	3.2	-	-	8	-	-	2	-	-	2	20	100	-	-	-	-
H5	30	3.2	-	-	8	-	1	-	-	-	2	20	100	-	-	-	-
H6	30	3.2	-	-	8	-	0.2	-	-	-	2	20	100	-	-	-	-
H7	30	3.2	-	-	8	-	2	-	-	-	2	20	100	-	-	-	-
M40	30	3.2	-	-	8	-	2		0.5		2	20	100	-	-	-	-
M41	30	3.2	-	-	8	1	-	-	0.5	-	2	20	100	-	-	-	-
M42	30	-	4.4	-	8	-	2	-	0.5	-	-	-	-	-	2	5	-
M43	30	-	4.4	-	8	1	-	-	0.5	-	-	-	-	-	2	5	-
M44	30	3.2	-	-	8	1	-	-	0.5	-	2	20	100	-	2	5	0.0015
M45	30	-	4.4	-	8	-	2	-	0.5	-	-	-	-	-	2	5	0.0015
M45b	30	-	4.4	-	8	-	2	-	0.5	-	-	-	-	-	2	5*	0.0015
M46	30	-	4.4	-	8	1	-	-	0.5	-	-	-	-	-	2	5	0.0015
M47	30	-	4.4	-	8	-	2	-	0.5	-	-	-	100	-	2	5	0.0015
M48	30	-	4.4	-	8	1	-	-	0.5	-	-	-	100	-	2	5	0.0015
M49	30	-	-	4.4	8	-	2	-	0.5	-	-	-	-	-	2	5*	0.0015

Table 1. Continued

M50	30	-	-	4.4	8	1	-	-	0.5	-	-	-	-	-	2	5*-	0.0015
M51	30	-	-	4.4	8	-	2	-	0.5	-	-	-	100	-	2	5*-	0.0015
M52	30	-	-	4.4	8	1	-	-	0.5	-	-	-	100	-	2	5*	0.0015
M53	30	3.2	-	-	8	0.5	-	-	0.1	-	2	20	100	0.3	-	-	-
M54	30	3.2	-	-	8	-	-	-	-	0.25	2	20	100	-	-	-	-
M55/ 55a	30	-	4.4	-	8	-	-	-	-	0.25	-	-	-	-	2	5*	0.0015
M55b	30	-	4.4	-	8	-	-	-	-	0.5	-	-	-	-	2	5*	0.0015
M55c	30	-	4.4	-	8	-	-	-	-	1.0	-	-	-	-	2	-5*	0.0015
M56	30	3.2	-	-	8	1	-	-	0.5	-	2	20	100	0.3	-	-	-
M57	30	3.2	-	-	8	1	-	-	0.5	-	2	20	100	0.3	2	-5*	0.0015
M58	30	-	4.4	-	8	-	2	-	0.5	-	-	-	-	0.3	2	-5*	0.0015
M59	30	-	2.2	-	8	-	-	-	-	-	-	-	-	-	2	5*	0.0015
M60	30	-	2.2	-	8	-	-	-	-	-	2	20	-	-	-	-	-
M61	30	3.2	-	-	8	0.1	-	-	-	-	2	20	100	0.3	-	-	-
M62	30	3.2	-	-	8	0.5	-	-	0.1	-	-	-	100	0.3	2	5*	0.0015
M63	30	3.2	-	-	8	1.0	-	-	0.5	0.25	2	20	100	-	-	-	-
M64	30	-	-	4.4	8	1.0	-	-	0.5	0.25	-	-	-	-	2	5*	0.0015
M65	30	-	-	4.4	8	1	-	-	0.5	-	-	-	-	0.3	2	5*	0.0015
M6	15	-	2.2	-	8	-	-	-	-	-	-	-	-	-	-	-	-
H8/ M54	30	3.2	-	-	8	-	-	-	-	-	2	20	100	-	-	-	-

H9	30	3.2	-	-	8	-	-	-	-	0.25	2	20	100	-	-	-	-
H10	30	3.2	-	-	8	-	-	-	-	0.5	2	20	100	-	-	-	-
H11	30	3.2	-	-	8	-	-	-	-	1.0	2	20	100	-	-	-	-
H12	30	3.2	-	-	8	-	-	-	-	2	2	20	100	-	-	-	-
H13	30	3.2	-	-	8	-	-	-	-	5	2	20	100	-	-	-	-
H14	30	3.2	-	-	8	-	-	-	-	7.5	2	20	100	-	-	-	-
H15	30	3.2	-	-	8	-	-	-	-	10	2	20	100	-	-	-	-
H16	30	3.2	-	-	8	-	-	-	-	-	2	20	100	-	-	-	-
H17	30	3.2	-	-	8	0.25	-	-	-	-	2	20	100	-	-	-	-
H18	30	3.2	-	-	8	0.5	-	-	-	-	2	20	100	-	-	-	-
H19	30	3.2	-	-	8	0.75	-	-	-	-	2	20	100	-	-	-	-
H20	30	3.2	-	-	8	1.0	-	-	-	-	2	20	100	-	-	-	-
H21	30	3.2	-	-	8	1.5	-	-	-	-	2	20	100	-	-	-	-
H22	30	3.2	-	-	8	2.0	-	-	-	-	2	20	100	-	-	-	-
H23	30	3.2	-	-	8	2.5	-	-	-	-	2	20	100	-	-	-	-
H24	30	3.2	-	-	8	3.0	-	-	-	-	2	20	100	-	-	-	-
M15	30	3.2	-	-	8	0.1	-	-	-	-	-	-	-	-	-	-	-

3.1.1. Media preparation

The non-heat labile chemicals: sucrose, Gamborg-B5 salts (Sigma, UK), Murashige and Skoog salts (MS, Sigma, UK), Cytokinins – 6-Benzylaminopurine (BAP, Sigma, USA), Kinetin (Kn, Sigma, USA); Auxins – α-Naphthaleneacetic acid (NAA, Sigma, USA) and other growth regulators – 1-Phenyl-3- (1,2,3-Thiadiazol-5-yl) Urea (TDZ, Sigma, USA) were weighed in the amount required for each protocol and put in labeled beakers. Distilled water was added and beakers were placed on a magnetic stirrer until the components were totally dissoloved. The pH of the solutions was adjusted to 5.6 adding a few drops of either Hydrochloric acid (HCl, 1N) or Sodium hydroxide (NaOH, 2N) solutions. The final volumes were adjusted and solutions transferred into labeled flasks. Then, 0.8% agar (BDH, England) was added into the solutions and the flasks were kept on the hot plate until boil. The flasks with boiled media were covered with aluminium foil, sealed with parafilm and put into an autoclave at 120^0C for about 20 minutes. The media were taken out and allowed to cool down for sometime. In the meantime, the laminar flow was cleaned using 70% alcohol and cotton. The previously autoclaved empty bottles were placed inside. Heat labile chemicals - Citric acid (CA, Sigma, USA), Adenine (Kodak, USA), Thiamine (Th., Fisher, USA), 2-isopentenyladenine (2-iP, Sigma, USA) and Gibberellic acid (GA_3, Sigma, USA) were put into the media while the media was still warm, using sterile membrane filters (0.22 µM pore size, Millex, USA; Flowpore, Germany). About 10ml of media were poured into each 39 ml

bottles and covered with the previously autoclaved lids in the laminar flow. Each bottle was labeled and kept refrigerated until required.

Stock solutions were prepared for BAP, NAA, TDZ, CA, Th, Ad, 2iP, GA_3, MS*, RT (8) and Vitamin B12 due to the small concentration to be used for the experiment (Table 2).

Table 2. Stock Solutions

Chemicals	Quantity required	Amount dissolved in distilled water to prepare stock solution	Stock solution per media
BAP[1]	0.1 mg/l	0.05 g/100 ml	200 µl/l
NAA[2]	0.1 mg/l	0.05 g/100ml	200 µl/l
TDZ[2]	2.5 mg/l	0.005 g/50 ml	2.5 ml/1
CA	100 mg/l	750 mg/15 ml	2 ml/1
Th	20 mg/l	0.05 g/25 ml	10 ml/l
Ad	2 mg/l	0.005 g/25 ml	10 ml/l
2iP[1]	2 mg/l	0.1 g/100 ml	2 ml/l
GA_3	0.3 mg/l	0.001 g/10 ml	3 ml/l
IBA[2]	5 mg/l	5 mg/100 ml	100 ml/l
MS* • Macronutrients o NH_4NO_3 (Sigma, USA) o KNO_3 (Sigma, USA) o $MgSO_4.7H_2O$ (Sigma, USA) o KH_2PO_4 (Sigma, USA) o $CaCl_2.2H_2O$ (Sigma, USA) • Micronutrients o $MnSO_4.H_2O$ (Aldrich, Germany) o $ZnSO_4.7H_2O$ (Aldrich, Germany) o H_3BO_3 (Sigma, USA) o KI (Sigma, USA)		1650 mg/l 1900 mg/l 370 mg/l 170 mg/l 440 mg/l 1690 mg 1060 mg 620 mg 85 mg 25 mg 2.5 mg 2.5 mg 745 mg 557 mg	± 400 ml/ l 10 ml/ l 2 ml/l

Table 2. Continued

o $Na_2MoO_4.2H_2O$ (BDH, England) o $CuSO_4.5H_2O$ (Sigma, USA) o $CoCl_2.6H_2O$ (Sigma, USA) o $Na_2EDTA.2H_2O$ (Sigma, USA) o $FeSO_4.7H_2O$ (Sigma, USA)			
RT (8)[3] • Thiamine HCl • Pyridoxal 5-phosphate[+] (Sigma, USA) • Biotin[+] (Sigma, USA) • Riboflavin[+] (BDH, England) • Folic acid[+] (Sigma, USA) • Nicotinic acid[+] (Sigma, USA) • Choline Chloride (Aldrich, Germany) • Calcium Pentothenate (Aldrich, Germany)	1 mg/l 1 mg/l 1 mg/l 0.5 mg/l 0.5 mg/l 2 mg/l 1 mg/l 1 mg/l	0.05 g/100 ml 0.05 g/100 ml 0.05 g/100 ml 0.025 g/100 ml 0.025 g/100 ml 0.1 g/100 ml 0.05 g/100 ml 0.05 g/100 ml	2 ml/l
Vitamin B12 (Sigma, USA)	1.5 µg/l	0.001 g/50 ml	75 µl/l

[1] Dissolved with few drops of HCl (1N)
[2] Dissolved with few drops of NaOH (2N)

MS* was prepared directly from chemicals by dissolving and mixing the listed chemicals. First, three separate stock solutions i.e. MS macronutrients, micronutrients and Iron/EDTA stock solutions were prepared. For MS macronutrients, each ingredient were weighed and dissolved one by one into a small amount of distilled water. Then the final volume of the mixture was adjusted to one litre. MS micronutrient stock solution was prepared in the same manner as macronutrients and finally adjusting the volume to 100ml. For the third stock solution, 745mg $Na_2EDTA.2H_2O$ was dissolved in some distilled water and heated just before boiling point. Then 557mg $FeSO_4.7H_2O$ was added slowly while stirring. Finally, the mixture was allowed to cool and the volume was adjusted to 100ml.

[3] To prepare RT (8) vitamin stock solution, the following chemicals (ingredients) have been dissolved alone and mixed together into the same 100ml of distilled water.

* Inositol* - those that have (*) refer to myo-inositol instead of inositol

3.1.2. Plant material

Three accessions of *Mucuna pruriens* (10084, 14880 and 15169) were obtained from the forage genebank collection at ILRI (Figure 1a). Seeds were scarified using sand paper and pre-germinated on trays covered by germination paper inside an incubator for about a week until they were transferred to pots filled with sterile soil composed of 3:2:1 ratio of soil, manure and sand, respectively. The plants grew in pots inside an incubator with maximum and minimum temperatures of 37/25°C with a 12/12 hours photoperiod for the thermotherapy treatments during three weeks (Figure 1b). Single node cuttings were made using scissors that were disinfected with 70% alcohol between cuttings of each plant (Figure 2a). The cuttings were dipped into small beakers filled with tap water and labeled with the respective accession and plant numbers.

(a) (b)

Figure 1. (a) *Mucuna pruriens* – virus infected plant material in a green house. (b) *Mucuna pruriens* seedlings ready for meristem extraction.

3.1.3. Surface Sterilization of Plant Material

The beakers were placed inside the laminar flow and the water drained holding the cuttings inside the beaker using forceps. The cuttings were rinsed with about 10 ml of 70% alcohol for 2 minutes, then transferred to autoclaved and numbered flasks containing about 100 ml of 10% commercial bleach. The flasks were covered with aluminium foil and placed in a shaker for 30 minutes. Finally, the cuttings were rinsed with sterile distilled water about 5 times until all the bleach was removed. To avoid browning of the tissues due to phenolic components, a citric acid solution (150 mg/l) was added after the last wash.

3.1.4. Culture Initiation

Meristems from both apical and axillary parts of the plant were extracted under a dissecting microscope (Carl Zeiss, West Germany) and cultured under aseptic conditions onto solidified

media (M29 or M35) contained in bottles (Figure 2b and c). The bottles were sealed and kept inside the incubator at $25^oC\underline{+}2^oC$ and a 12/12 hours photoperiod. Subsequently, the initiated meristems were subcultured after every two to three weeks onto fresh media (Figure 2d).

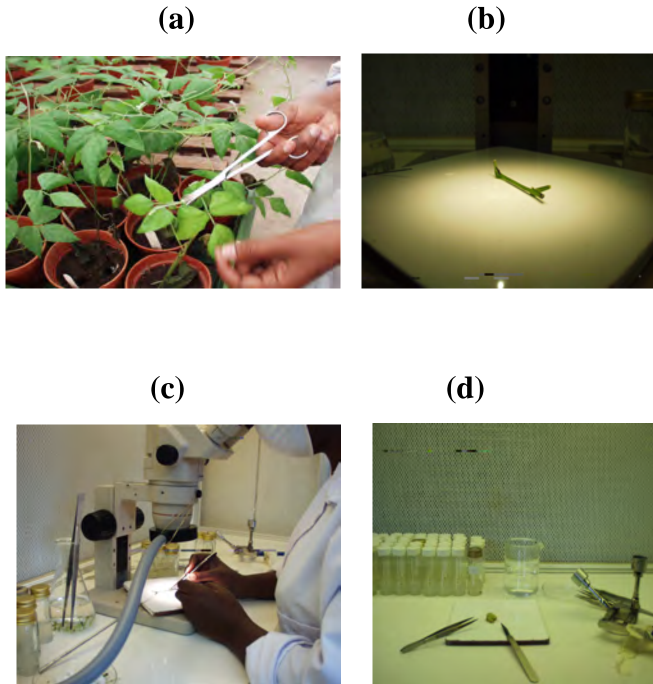


Figure 2. (a) Plant stem containing 2-3 nodes in about three weeks after planting. (b) Sterilized cutting ready for meristem extraction. (c) Meristem extraction under dissecting microscope inside a laminar flow cabinet. (d) Callus sub-culturing

3.2. Data recording and measurements

The response of different media combinations and accessions were recorded every one, two and/or three weeks from the day of subculture. Observations were made on color, size, type of tissue, contamination, previous tissue and comments. The data was recorded using code symbols for the different characteristics: Color (Green – G=1, Pale Green – PG=2, Yellow/Brown/Black – Y/BW/BL=3); Size (Very Small – VS=0.5, Small – S=1, Medium – M=2, Large – L=3), the approximate size range for the different size classes are 0-5 mm (VS), 5-10 mm (S), 10-15 mm (M), 15-20 mm (L) and 20-25 mm (VL); Type of explant tissue (Callus=1, Shoot=2, Leaves=3); and Contamination (Bacteria=1, Fungus=2, Phenolic=3). The code letters were used to make the initial recordings and later on, these

were changed into number codes to facilitate the statistical analysis. Color was a good indication of survival of both types of tissues (callus or shoots) in each media. Green and pale green tissues were considered alive for survival purposes and percentage was calculated over total as: $[(G+PG)/(G+PG+BW+Y)] \times 100$. After completion of each experiment, a preliminary analysis was done and the media showing higher survival percentage were selected and used along with new media combinations for subsequent experiments. Each treatment had varying numbers of replicates in different experiments due to mortality of plants.

Experiments were checked often for contamination of bacteria and fungi and rescued using 10% commercial bleach and 0.25 mg antibiotic (tetracycline) per 100 ml of distilled water. Those tissues that could not be rescued were discarded and replaced by other similar plants, when possible.

3.3. Experimental layout

3.3.1. Experiment 1

The main objective of this first experiment was to compare the effects of three major cytokinins (BAP, 2-iP and Kn) on survival and type of explant tissue (callus and shoots) of *Mucuna pruriens*. Three concentrations each of 2-iP and Kn i.e. 0.5, 1, 2 mg/l and 0.2, 1, 2 mg/l respectively, were evaluated, while BAP 0.1 mg/l was used as control (H1 – H7, Table 2). Previous data from ILRI indicated this concentration of BAP as being favorable for *Mucuna* tissue culture growth. The vitamins adenine, thiamine and citric acid stock solutions (Table 1) were added into all media (as per ILRI previous data recommendation). A total of 130 tissues (a mixture of all available shoots and callus) were used as starting explant materials in almost equal proportion for each accession. The experiment was arranged as factorial completely randomized design (CRD) with three replications. Factor A was treatments (media) and Factor B accession. Factor A consisted of seven levels (media combinations – H2 up to H7 and control) and Factor B had three levels (accessions) *viz*; 10084, 14880 and 15169. Observations were taken at one, two and three weeks of subculture.

3.3.2. Experiment 2

In this experiment alternative media recommendations from a recent paper on *Mucuna* tissue culture were tested. Two cytokinins i.e. either BAP 1 mg/l or 2i-P 2 mg/l in combination with an auxin – NAA 0.5 mg/l (Chattopadhyay *et al.,* 1995) with two sets of vitamins either C.A. + Adenine + Thiamine with B5 basal media (in use at ILRI) or RT (Kaul and Staba, 1968) with MS basal media (Sigma, USA) were used (M40-43, Table 1&2). Due to the unavailability of 3 of the RT vitamins, this experiment had only 5 of the 8 recommended vitamins prepared in one single stock solution. The full RT vitamin complex is composed of: Thiamine HCl, Pyridoxal 5-phosphate, Biotin, Riboflavin, Folic acid, Nicotinic acid, Cholin chloride, Calcium pantothenate prepared in one stock and Inositol (Fisher, USA) plus vitamin B12. In this experiment the vitamins Nicotinic acid, Cholin Chloride and Calcium Pantothenate were not available, so they were not included. H1/M32 was the control. A mixture of both types of tissues (a few shoots and the rest, mostly callus) that had survived from experiment I was used for culture. The experiment was replicated three times with unequal units of treatments per accession and replication.

3.3.3. Experiment 3

Separate stock solutions of RT(8) (all the 8 recommended vitamins) and MS* basal media from individual chemicals were prepared. M45-48 were set to contain MS basal media with cytokinin BAP/2-iP and an auxin NAA; with or without CA; and the RT(8) vitamins plus Inositol and Vitamin B12. On the contrary, M49-52 were composed of MS* basal media with similar composition of growth regulators; with or without CA; and same RT(8) vitamins plus Vitamin B12, but myo-inositol instead of inositol. The already available media M32 without NAA and M41 with NAA, and M44 similar to M41 but with the whole vitamins stock solution were also included. The concentration of BAP was also different i.e. 0.1mg/l. The aim here was to compare different treatment (media) combinations simultaneously; like ready-made MS versus MS*, BAP versus 2iP, previously used vitamins (Ad and Th) versus the new vitamins (RT(8)+vitB12) and with and without CA. The experiment was designed as factorial CRD with three replications, each replicate with five units (bottles). Factor A was treatment (media) with 12 levels while Factor B was accession with 2 levels. There was a shortage of tissues from accession 10084, due to low survival from earlier experiments,

hence only two accessions *viz*; 14880 and 15169 were used. Only callus were used for cultures in this experiment while shoots were cultured separately in another experiment (4).

3.3.4. Experiment 4

Since we lost most of the shoots after culturing the 2nd experiment, new meristems were extracted from all the three accessions and initiated in shoot inducing media (M29 and M35). These new shoots along with a few remnant shoots from experiment 2 were grown onto the same media used in experiment 3 along with M29 and M35 (M29, M35, M32, M41, M44-M52). The experiment was conducted non-replicated because of lack of enough shoots required for twelve treatments (media). We used 33 units per treatment M29 and M35, meaning subculturing onto the same media; 22 units in M32 and about 5 units for the rest of the media.

3.3. 5. Experiment 5

In this experiment, 13 media (Table 1&2) were evaluated. The media was composed of treatments with half strength basal media and sucrose but no growth hormones (M6); three types of basal media: MS – full (M55) and half strength (M59 and M60), MS* (M64 and M65) and Gamborg B-5 (M29, M32, M53, M54, M61, M62 and M63). Various hormones (cytokinins – BAP and 2-iP, auxin – NAA, other growth regulator – TDZ and GA_3) and vitamins (Ad., Th.e, CA and RT (8), vitamin B12 and myo-inositol) were used in this treatments. The objective here was to test the new growth hormone, TDZ along with a wide range of media types. Only callus were used as culture material, due to shortage of shoot tissues. The experiment was replicated 3 times with 14 units per each treatment.

3.3.6. Experiment 6

Some commonly used media from earlier results at ILRI were used as control (M29, M32, M35) and selected media from experiments 4 and 5 were included in this experiment (M6, M41, M44-M65, Table 2). Both shoots from the newly initiated meristems and the surviving shoots from experiment 4, were used for this experiment. The shoots were cultured for two weeks (2WAC) and then sub-cultured again into the same media and grew for two more weeks.

3.3.7. Experiment 7

This experiment had 17 media treatments (Table 1&2). The treatments consisted of: half strength basal media and sucrose but no growth hormones (M6); three types of basal media (MS – full (M45, M45b, M48, M55 and M58) and half strength (M59 and M60), MS* (M64 and M65), Gamborg B-5 (M53, M54, M56, M57, M61, M62, M63). The treatments had various combinations of hormones (cytokinins – BAP and 2-iP, auxin – NAA, other growth regulator – TDZ and GA_3) as well as different combinations of vitamins – Adenine, Thiamine, CA and RT (8), B12 and myo-inositol. These were evaluated simultaneously. The source of explants for this experiment was callus from experiment 5 and some recently raised cultures (only callus) of mixed accessions. The design was CRD with three replications having 5 units per each media per replication.

3.3.8. Experiment 8

In experiment 6, media containing TDZ 0.25mg/l (M54 and M55) showed better survival percentage. To evaluate the efficacy of TDZ two additional concentrations (0.5 and 1.0 mg/l) in media M55b and M55c were included. Further to confirm the results of experiment 6, many of the media (M32, M35, M44, M45, M55a, M60, M61, M65, Table 2) that responded well were also repeated in this experiment. Experiment 8 had only shoots tested in a total of ten treatments. Due to the limited numbers of shoots produced from experiment 6 (the source of shoots for this experiment), there was not enough tissues for replications and the numbers of shoots per accession and per treatment were not uniform. However, this experiment was used to make preliminary evaluations for the different treatments.

3.3.9. Experiment 9a

Based on preliminary observations from experiments 6 and 8, it was found that media containing TDZ showed better performance for survival. So, more exploration on the performance of TDZ was undertaken. Accordingly, seven concentrations *viz;* 0.25, 0.5, 1.0, 2.0, 5.0, 7.5, 10.0 mg/l in M54 media were evaluated. Media containing these concentrations were named as H9 to H15, where M54 and H9 were the same. Media H8 without TDZ and M32 were included as controls (Table 2). Callus obtained from Experiment 7 were sub-cultured into these media three times every 2 weeks. The first subculture consisted of three replications; accession 14880 in replication I; accessions 14880 and 15169 in replication II

and all the three accessions (10084, 14880 and 15169) mixed in replication III. The second subculture consisted of a mixture of all three accessions and was replicated 4 times in CRD. The third subculture consisted of only two accessions (14880 and 15169) each in different replication. Six units (bottles) were used in each treatment for subculture I and II, while for subculture III, a total of 10 units was used.

3.3.10. Experiment 9b

The above experiment (9a) 3rd subculture was repeated by culturing accession 14880 and 15169 in same media separately. In this experiment, more detail measurements were taken for the first time in order to try to better quantify the responses of the TDZ treatments. Hence, the callus was also quantified by weight (mg) and size (mm) in addition to the previous observations of color, size in three categories (small, medium and large). Observations were taken at two, three and four weeks after sub-culturing, weighing the whole bottles with the callus inside. After final observations on the fourth week, the calli were taken out of the bottles, drained on a filter paper and weighed again.

(a) **(b)** **(c)**

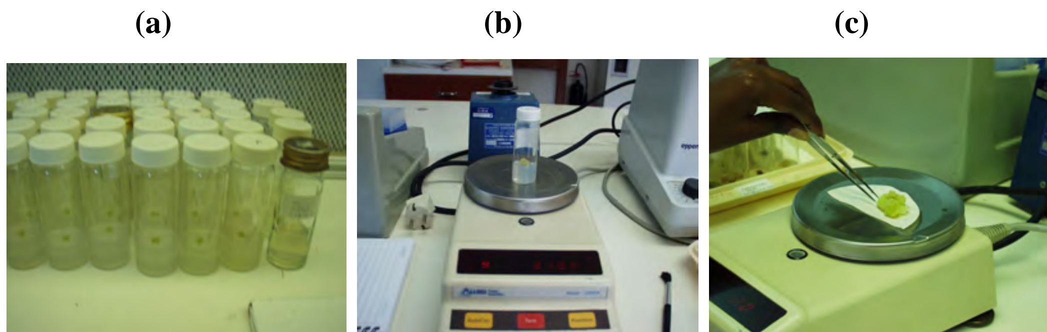


Figure 3 (a) Callus sub-cultured of approximately equal size. (b) Weighing of callus inside a bottle. (c) Weighing of callus outside its bottle after drying the excess media on a filter paper.

3.3.11. Experiment 10

In all our preceding experiments, the meristem tissues were cultured with the main objective of regenerating virus free shoots. However, the shoot proliferation and elongation was not satisfactory while the callus failed to promote shoots even in media previously used successfully for *Mucuna pruriens* micropropagation though with different genotypes and

types of explant tissues (they used epicotyl or hypocotyls plus cotyledons). So we used their protocol to check if the failure was due to our methodology. We used different explants such as shoot apex, epicotyl and hypocotyls plus cotyledon for culturing this experiment instead of meristems. To obtain these explants, seeds were germinated on germination paper in a tray. After germination, the above-mentioned explants were excised and sterilized using the same procedure adopted for meristems (ILRI procedures). Media was poured into jam jars and the explants were planted. Due to high contamination, mainly with fungi, the experiment was discarded and repeated with a different approach, following exactly their protocol. Then, the seeds were first sterilized and then cultured onto media containing 30% sucrose and 8% agar. Test tubes containing 20 ml media instead of the small bottles were used, in order to optimize space. Most of the media turned brown and the seeds did not grow. The few seeds that germinated were cultured on M49 & M50 media (Table 2). Despite all the efforts, no satisfactory results were obtained, since all tissues turned brown due to phenolics and died and also were heavily contaminated mostly with fungi.

3.3.12. Experiment 11

One shoot of large size from experiment 8 was obtained from M55 medium containing TDZ at 0.5mg/l. This shoot was used to promote roots by adding another medium containing half-strength liquid Gamborg-B5 supplemented with IBA (1mg/l). The half strength Gamborg-B5 liquid medium was added on top of the existing M55 solid medium. The plant was then sub-cultured into a fresh media without IBA (M23), after 5 days.

3.3.13. Experiment 12

This experiment was conducted to investigate the effect of TDZ on rooting. For rooting, shoots grown in media with and without TDZ from experiment 8 and new shoots developed from meristems were taken and cultured on growth regulator free medium (M23, Table 2). A few drops of liquid rooting media containing ½ B5 plus an auxin IBA (1mg/l) were then added on top of the solid media, and remained for 5 days. Cultures were then sub-cultured into media M55a and M55b and remained for two weeks.

3.3.14. Experiment 13

Our previous results revealed better survival performance of shoots as well as callus on media containing BAP. So, this experiment was designed to re-confirm the response of different BAP concentrations. Nine concentrations *viz;* 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l of BAP in M32 medium were tried. These media were marked as H16-H24 (Table 1); M15 (same as M32 but without vitamins) and M32 containing BAP (0.1 mg/l) were both used as controls. The experiment was divided into two parts: 13a and 13b.

In 13a, newly excised meristems from all the three accessions were cultured to induce shoots and callus. There were 124 different meristems obtained from 38 plants used in this experiment. Shoots were then used for further elongation while callus were cultured to develop shoots. In 13b, only uniform large older callus of the same three accessions from previous experiments were used. Callus cultures in each accession have almost been subcultured from the same plant. In both experiments, the three accessions: 10084, 14880 and 15169 were replicated twice each having 2-3 units.

4. RESULTS

4.1. Meristem Culture to Induce Callus and Shoots

Meristem explants were cultured on M29 and M35 (Table 1) media to induce callus and shoots. Both types of media induced callus and shoots within two to three weeks after meristem extraction. Calli were easily induced but they did not develop into shoots, later on. Both calli and shoots were subcultured onto fresh media after every two weeks and could be maintained for longer periods for subsequent use. The shoots survived better if transplanted frequently (i.e. every two weeks). The calli were able to stay alive for a month or even longer periods without collapsing (i.e. turning brown or black and drying), growing fast into large clumps of bright green cells. Since we induced plenty of calli, more experiments were conducted using callus than shoot cultures. Different color gradients of callus were observed ranging from green, pale green to yellow, white, brown and black. Only the green and pale green callus (fig 4a and b) were subcultured. The remaining color grades were discarded due to degeneration into dead tissues mostly due to phenolic exudation. We have tried to diminish this browning effect by incorporating citric acid in the media as well as putting the cuttings into a citric acid solution (150mg/l of sterilized distilled water) while waiting for the meristem extraction (after removing the last water of the sterilization process).

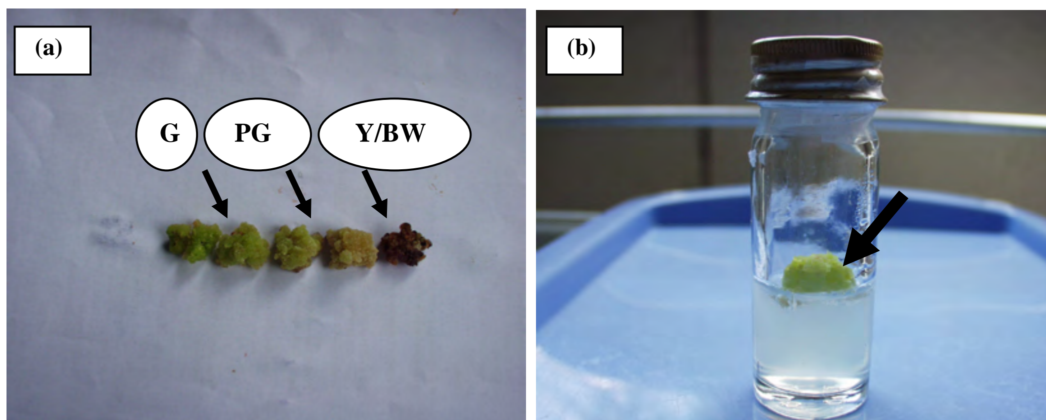


Figure 4. Callus grown *in-vitro* **(a)** G=Green→PG=Pale green → Y/BW=Yellow/Brown respectively. **(b)** Green callus

4.2. Callus Survival and Organogenesis in Nutrient Media

In the 1st experiment, one to three different concentrations of three types of cytokinins were evaluated: BAP (0.1mg/l), 2iP (0.5, 1 and 2 mg/l) and Kn (0.2, 1 and 2 mg/l). Seven media (H1-H8, Table 1) were prepared keeping all other components (nutrients, sucrose, agar and vitamins) constant. A mixture of both types of tissues (callus and shoots) was cultured onto these media. Initially, a few shoots elongated in these media but the callus remained undifferentiated. In the three week period callus increased in size but tissues started diminishing affecting survival. Accession 14880 was the best in survival percentage in all the three weekly observations, on consecutive weeks. The analysis of variance for percentage of survival at one, two and three weeks after culture (WAC), revealed significant differences amongst accessions (genotypes) but not amongst media (Table 3). The survival percentage decreased in the 2nd and 3rd WAC in all accessions except on accession 15169, which showed an increase at 3rd WAC. Accession 15169 improved in performance due to the replacement of highly infected cultures after the 2nd week. This was done in order to keep the sample size constant. Accession 14880 had significantly higher survival rates in the first and second weeks. At three weeks, both accessions 14880 and 15169 had significantly higher survival rates than accession 10084 (Table 4). The different media types had a mean survival rate ranging from 43% to 59% in the 1st WAC, 29% to 49% in the 2nd WAC and 19% to 49% in 3rd WAC.

Table 3. Analysis of variance for survival percentage of callus and shoot cultured tissues (Experiment 1).

Source	df	MSS		
		1st WAC	2nd WAC	3rd WAC
Rep	2	6476.78	676.37	1906.27
Media	6	415.28	490.76	883.80
Accession	2	10687.67**	6107.67**	2210.25**
A*B	12	275.48	625.97	656.75
Error	40	772.68	482.74	633.42

**Significant at 1% level of significance
WAC: Weeks after culture

Table 4. Survival percentage of tissues (green and pale green callus and shoots) of different accessions at different weeks after culture (Experiment 1).

Accession	Survival*		
	1WAC	2WAC	3WAC
10084	41.27^{b}	26.98^{b}	26.98^{b}
14880	78.57^{a}	59.13^{a}	46.83^{a}
15169	37.94^{b}	33.17^{b}	41.43^{a}

CD (Critical Difference)=17.342(1WAC), 13.707(2WAC), 15.702(3WAC); WAC:Weeks after culture;*: Average over replications. Means in a column with same superscript are not significantly different from each other at 5% level.

In experiment 2, the cytokinins BAP and 2iP plus an auxin (NAA) as well as two sets of vitamins (Ad&Th versus RT&VitB12) and basal media salts were evaluated. Four media (M41-M43, Table 1) were developed and media H1/M32 was used as control (Table 1). The shoots and callus that survived in experiment 1 were cultured into this experiment. The survival percentage was higher (about 80%) in media M41 and M43. Both media had 1 mg/l of BAP plus NAA but differed in their salt and vitamin content. M40 and M42 recorded about 60% survival, very close to the control media (M32). These media had 2iP plus NAA in common. M32 was similar to M41 except on the concentration of BAP (M41 had 1 mg/l of BAP) and NAA (M32 had no NAA). From a total of 50 shoots cultured in these media, 14 turned into a callus phase, while 36 elongated and could be maintained as shoots. When we look at the trend of shoots turning into callus, most of the shoots that turned into callus were in media M43 whereas M32 had the least numbers of shoots turning into callus. The callus did not differentiate into shoots. Since we had an unequal number of plants per each treatment (media), we could not analyze the data statistically, but this trend showed M32 has the best media to maintain most shoots alive, which is our main target in order to further obtain fully developed plants.

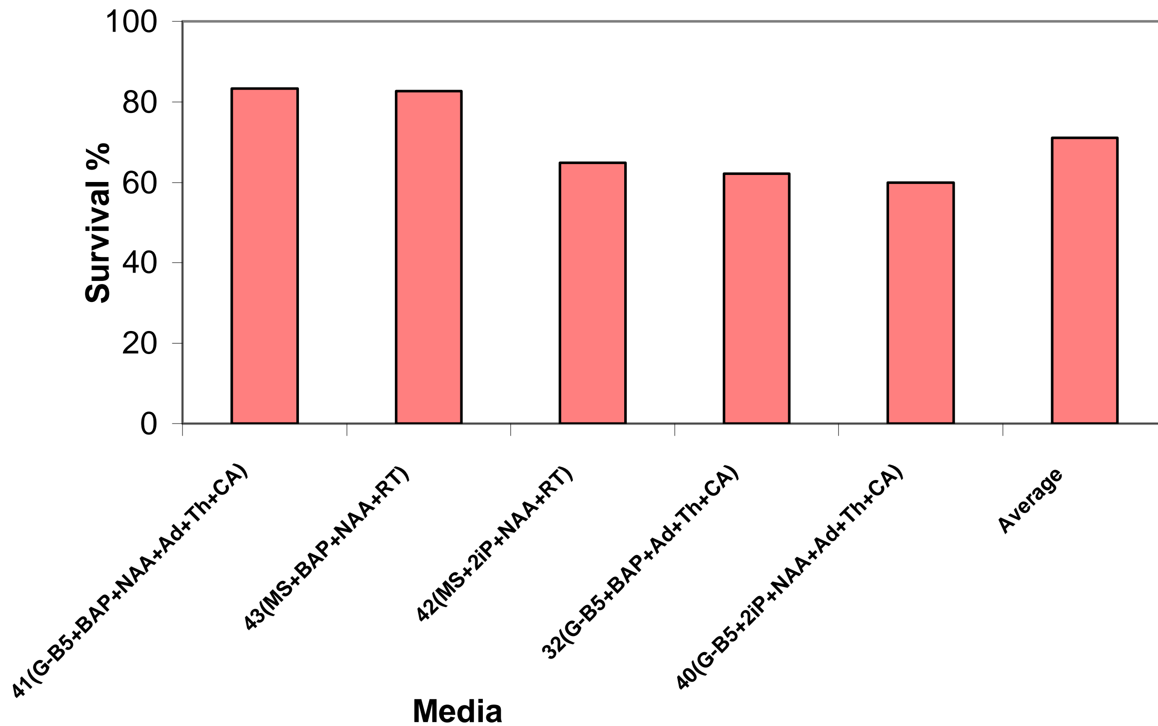


Figure 5. Effect of different media on survival percentage of callus and shoot tissues (Experiment 2).

In experiment 3rd and 4th, 12 media were prepared with different combinations of cytokinins, auxins, vitamins and salts. This evaluation had the main objective to compare ready made MS versus MS*, BAP versus 2iP, previously used vitamins (Ad and Th) versus the new vitamins (RT(8) + vitB12) and with and without CA simultaneously. We wanted to try this new vitamin set along with growth regulators since it worked well in an earlier report on *Mucuna pruriens* (Chattopadhyay *et. al.,* 1995). So we prepared a stock solution from 8 different chemicals (Table 2). Experiment 3 was only planted with callus while the experiment 4 was only planted with shoots. Only accessions 14880 and 15169 were used in these experiments due to lack of suitable plants of accession 10084. The main objective of experiment 3 was to induce shoot organogenesis *via* callus, while the 4th experiment had the main objective of inducing shoot multiplication and elongation, from previous shoots. Observations at 2 WAC revealed that the callus showed better survival percentage than the shoots, i.e. it grew bigger and green, but no organogenesis was observed. The analysis of variance for survival exhibited significant ($P \leq 0.01$) differences within media and accessions. However, there was no significant interaction between media and accessions (Table 5).

Accession 15169 showed significantly better callus survival percentage than 14880 (Table 6). The mean callus survival (average of the two accessions) in the various media ranged from 60% in M52 to 93% in M45b (Table 7). All media, except M45, M47, M51 and M52 (these had significantly lower survival rates), resulted in more than 75% callus survival. All the media that provided a high survival of tissues had either MS, MS* or G-B5 mainly with BAP (except M45b that had 2iP*) whereas the others had mostly 2-iP, except M52, that had also BAP regardless of the different salts and vitamins. From these results, the use of 2iP seems to be detrimental for tissue survival. The addition of NAA in BAP containing media improved the survival of callus cultured tissues.

Table 5. ANOVA for survival percentage of tissue from callus cultures (Experiment3).

Source	df	MSS
Rep	2	768.06
Media	11	691.54 [**]
Accession	1	2112.50[**]
A*B	11	369.33
Error	46	291.97

[**]Significant at 1% level of significance

Table 6. Survival percentage of callus of different accessions at 2 weeks after culture (Experiment 3).

Accession[**]	Mean survival
14880	71.94[b]
15169	82.78[a]

CD at 5%= 8.109

Table 7. Survival percentage of callus cultured tissues in different media at 2 WAC (Experiment 3).

Media[**]	Basal salt	Growth regulators	Vitamins	Mean survival
M44	G-B5	BAP+NAA	Ad+Th+ RT(8)+vit B12+CA	90.00[a]
M41	G-B5	BAP+NAA	Ad+Th+CA	79.17[abcd]

M32	G-B5	BAP*(0.1)	Ad+Th+CA	75.83[abcd]
M48	MS	BAP+NAA	RT(8)+vit B12+CA	86.67[ab]
M46	MS	BAP+NAA	RT(8)+vit B12	80.00[abc]
M50	MS*	BAP+NAA	RT(8)+vit B12	86.67[ab]
M52	MS*	BAP+NAA	RT(8)+vit B12+CA	60.00[bcd]
M45b	MS	2iP*+NAA	RT(8)+vit B12	93.33[a]
M45	MS	2iP+NAA	RT(8)+vit B12	70.00[bcd]
M47	MS	2iP+NAA	RT(8)+vit B12+CA	66.67[bcd]
M49	MS*	2iP+NAA	RT(8)+vit B12	76.67[abcd]
M51	MS*	2iP+NAA	RT(8)+vit B12+CA	63.33[bcd]

CD at 5%= 19.862

Means in a column with same superscript are not significantly different from each other at 5% level.

BAP*= All the media with BAP except M32 had concentration of 1mg/l , where it had 0.1mg/l.

2iP*= with an additional chemical (riboside).

MS*= MS basal media prepared from chemicals

The propagation of only shoots, experiment 4, consisted of newly induced shoots of three accessions and the old remnant shoots from experiment 2. Shoot elongation and survival percentage of tissues was recorded at 2 WAC. Due to insufficient equal number of shoots (due to high bacterial and fungi contamination after previous culture), these could neither be cultured equally in each media nor replicated, hence statistical analysis was not carried out. More shoots were cultured into M32, M29 and M35 (control and shoot inducing media) since we were afraid of losing the shoots in the newly tested treatment combinations. However, trends with total numbers of shoots cultured and elongated in different media is given in Table 8. Percentage of elongated shoots varied from 20% in M51 to 75% in M49. M49 recorded the highest elongation percentage followed by M45 (50%).

As for survival of shoots, M45 and M48 exhibited the maximum (100%) values, followed by M35 (80%) while M47 recorded the least (22%) (Figure 6). The media M29, M32, M35, M44, M45 and M48 have shown survival of tissues above the average. Although there were not enough numbers of plants to perform a statistical analysis, this media that had higher survival rates of elongated shoots indicated a better suitability to maintain and propagate green shoots, which is more important for us than callus survival. Many of the shoots that survived and elongated here in the new tested treatments, did turn brown and die later on. When combining both results of shoot survival (Figure 6) and shoot elongation (Table 8), we

selected M45, M32, M44, M35 and M29 as the best media combination in order to obtain more and larger green shoots.

Table 8. Percentage of cultured shoots elongated (Experiment 4).

Media	Basal salts	Growth Regulators	Vitamins	Number of cultured shoots	Number of elongated shoots**	% of elongated shoots
M49	MS*	2iP+NAA	RT(8)+vit B12	4	3	75
M45	MS	2iP+NAA	RT(8)+vit B12	4	2	50
M32	G-B5	BAP*	Ad+Th+CA	22	10	46
M44	G-B5	BAP+NAA	Ad+Th+RT(8)+vit B12+CA	7	3	43
M50	MS*	BAP+NAA	RT(8)+vit B12	7	3	43
M41	G-B5	BAP+NAA	Ad+Th+CA	5	2	40
M29	G-B5	BAP*+NAA* +GA_3	CA	33	11	33
M35	G-B5	BAP*+NAA +GA_3	CA	33	11	33
M46	MS	BAP+NAA	RT(8)+vit B12	3	1	33
M52	MS*	BAP+NAA	CA+RT(8)+vit B12	3	1	33
M47	MS	2iP+NAA	CA+RT(8)+vit B12	4	1	25
M48	MS	BAP+NAA	CA+RT(8)+vit B12	4	1	25
M51	MS*	2iP+NAA	CA+RT(8)+vit B12	5	1	20

BAP*= All the media with BAP except M32, M29 and M35 had concentration of 1mg/l, where M32 had 0.1mg/l, M29 and M35 had 0.5mg/l.
MS*= MS basal media prepared from chemicals.
NAA*= NAA concentration in M29 was 0.1mg/l but the rest had 0.5mg/l.
** = The total number of larger shoots

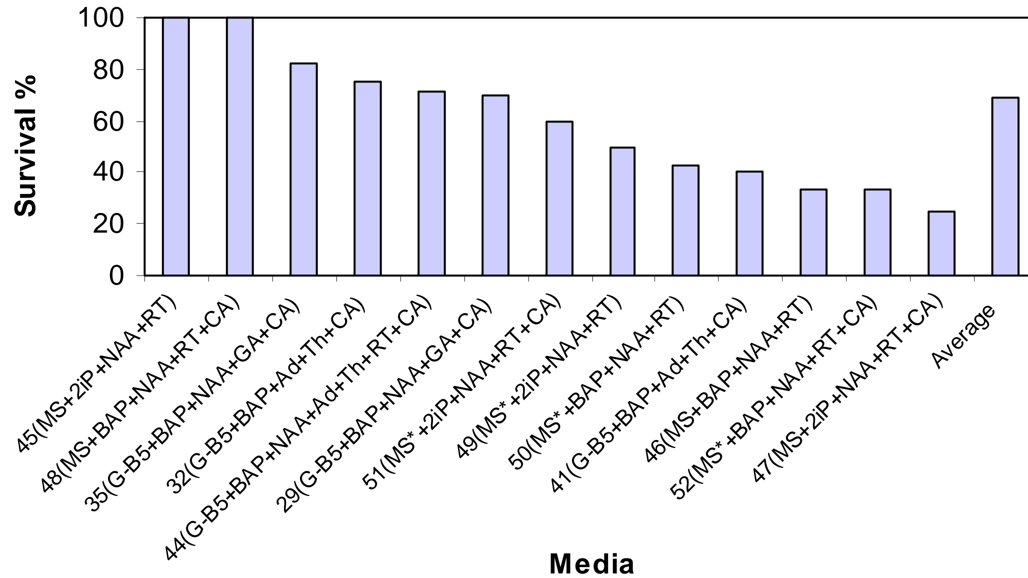


Figure 6. Effect of different media on survival percentage of shoot cultured tissues (Experiment 4).

New media combinations tested in experiment 5 contained growth regulators like TDZ and GA_3 in addition to the other components used in preceding experiments. This experiment had only callus tissues, growing in 13 types of media. Here, again the numbers of plants were too small and scattered through the different treatments due to high contamination rates, therefore it was not possible to do a statistical analysis of the data. However, trends in the results revealed maximum (100%) callus survival at 2WAC in M54 followed by M62, M55, M63, M29 and M6. The range varied from zero in M61 to 100% in M54 with an average of 42%. Low survival in media M32, M53, M59 and M61 was due to occurrence of contamination (bacteria and fungi) in cultures. From this data we can conclude that the addition of TDZ, specially with Gamborg-B5, did improve survival of green callus. Both ILRI vitamins (Ad, Th and CA) or RT, performed better when added into Gamborg-B5 salts.

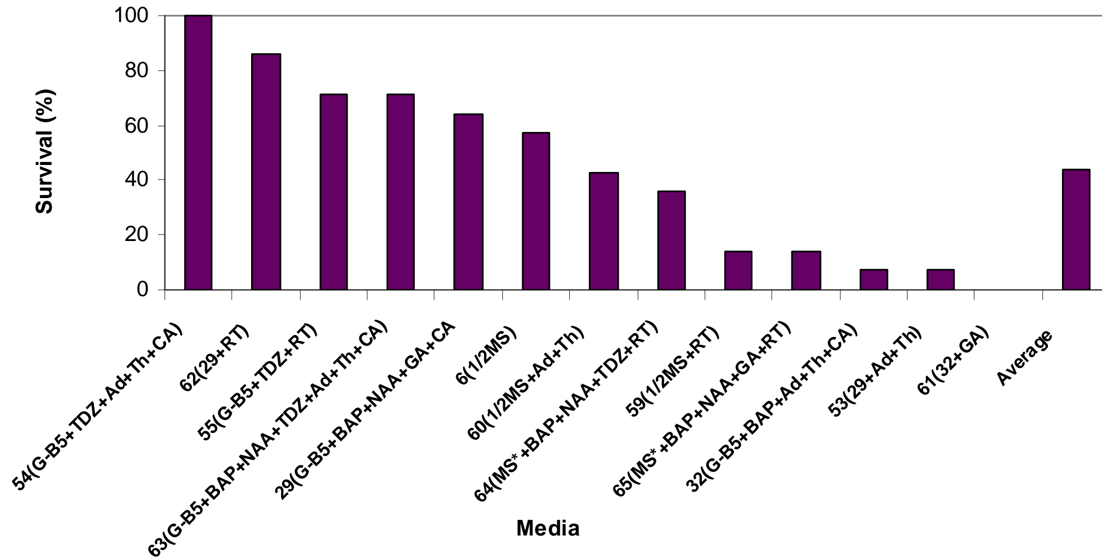


Figure 7. Effect of different media on survival percentage of callus cultured tissues (Experiment 5).

Experiment 6 had only shoots being cultured into the same treatments as experiment 5, plus several more. They were subcultured every week during four weeks, to avoid losing any shoot due to tissue degeneration and to also test as many media types as possible. The aim was to promote/elongate the existing shoots. Here, 27 media some commonly used as control (M32, M29 and M35) and others selected from experiments 4 and 5, were evaluated. In the beginning, 80 shoots were cultured, the majority of them stayed alive for 21 days but started to die thereafter. After a month, only 50 shoots, mostly small were still alive. A few multiple shoot initiation (Fig 8b) was observed in media containing TDZ (0.25 mg/l) growth regulator (M54 and M55a) (Data not shown). Subsequently, in experiment 8, two more concentrations of TDZ (0.5 and 1.0 mg/l) were investigated in M55b and M55c. Some of the media that responded well in experiment 6 were also repeated. However, the cultured shoots were old (3 months) and did not respond well. Only five of the twenty shoots sub-cultured from experiment 6 in the beginning could survive. Young shoots that were sub-cultured in similar media showed better survival. An average of approximately 75% survival was recorded. Media M35, M61, M55a and M55b performed higher than the average. M44, M45, M60, and M65 were similar to the average, while M32 and M55c were below average (Fig. 8a). The size of the shoots was larger in the better performing media. At 2nd WAC, these shoots were again sub-cultured onto the same media containing various amounts of TDZ growth regulator

(0.25, 0.5 and 1.0 mg/l). Medium with 0.5mg/l TDZ gave the best response (Figure 8b). We were also able to obtain one elongated shoot with leaves. This shoot was large enough to try rooting (Figure 9). Consequently, a root induction experiment (Experiment 11) was carried out. The enlarged shoot on medium with 0.5mg/l of TDZ was supplied with ½ G-B5 rooting liquid medium (where 1mg/l IBA was included instead of BAP) (Data not shown). Unfortunately, it failed to induce rooting. In order to investigate the effect of TDZ growth regulator on root induction, the left over larger shoots from TDZ containing medium and shoots from media without TDZ were further cultured (experiment 12). These shoots were all equally cultured in a solidified media without hormones (M23), supplemented with a few drops of liquid rooting media poured on top of it. However, still no rooting was observed (Data not shown).

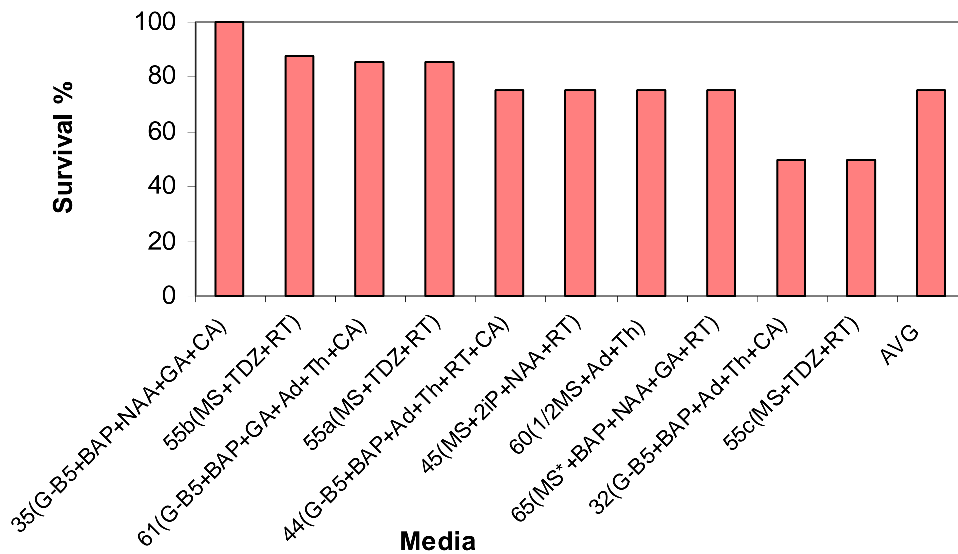


Figure 8(a). Survival of Shoot Cultured tissue in different media (Experiment 8).

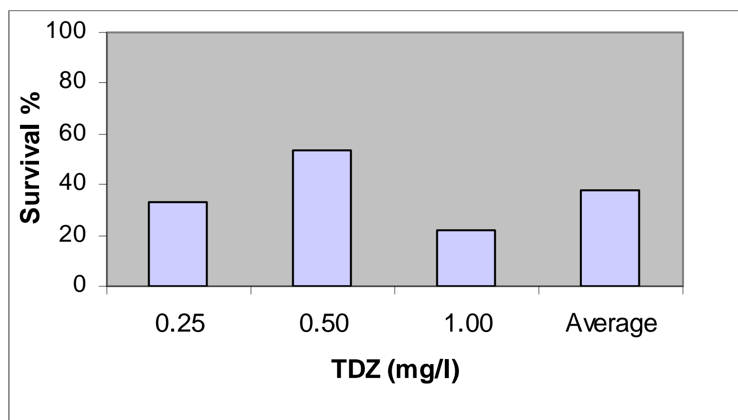


Figure 8(b). Response of three different concentrations of TDZ growth regulator for survival of shoot cultured tissues (Experiment 8).

Figure 9. Elongated shoot on TDZ containing media (Experiment 6).

Experiment 7 consisted of 17 media that were mostly used in experiment 5. Due to the shortage of shoots, only callus were used. Statistical analysis revealed that treatments differed significantly. A wide range of response on callus survival was observed, ranging from 83.33 % in M56 to 8.67 % in M6. Media M56, M35, M57, M53, M54, M55, M58, M60, M63, M32, M29, M63 were statistically similar. When examining the chemical differences in each treatment combination, we can conclude that treatments with MS* did not perform well (M64 and M65). Half strength MS alone (M6) or with RT vitamins (M59) did not give high survival of callus either. Although, when ILRI vitamins were added (M60) callus survival was about 50%. The media that gave higher callus survival, included G-B5, BAP, NAA, GA_3, CA, Ad and Th (M35, M32, M53, M56 and 57). The addition of vitamins RT only did well in combination with TDZ (excluding other hormones) (M55) or with 2iP (M58). TDZ seems to perform better alone in general, either in MS plus RT (M55) or G-B5 plus ILRI vitamins (M54).

Table 9. ANOVA for survival percentage of tissue from callus cultures (Experiment 7).

Source	df	MSS

Rep	2	450.28
Media	16	1264.09**
Error	32	588.38

**Significant at 1% level of significance

Table 10. Survival percentage of callus cultured tissues in different media at 2 WAC (Experiment 7).

Media	Basal salt	Growth regulators	Vitamins	Mean survival
M29	G-B5	BAP*+NAA*+GA_3	CA	43.00[abcdef]
M35	G-B5	BAP*+NAA+GA_3	CA	80.00[ab]
M53	G-B5	BAP*+NAA*+GA_3	Ad+Th+CA	70.00[abcd]
M56	G-B5	BAP+NAA+GA_3	Ad+Th+CA+	83.33[a]
M57	G-B5	BAP+NAA+GA_3	Ad+Th+CA+ RT(8)+vit B12	78.33[abc]
M62	G-B5	BAP*+NAA*+GA_3	CA+ RT(8)+vit B12	36.33[def]
M61	G-B5	BAP+GA_3	Ad+Th+CA	42.67[bcdef]
M32	G-B5	BAP*	Ad+Th+CA	57.33[abcde]
M54	G-B5	TDZ	Ad+Th+CA	59.89[abcde]
M55	MS	TDZ	RT(8)+vitB12	61.33[abcde]
M63	G-B5	BAP+NAA+TDZ	Ad+Th+CA	48.33[abcdef]
M64	MS*	BAP+NAA+TDZ	RT(8)+vit B12	26.67[ef]
M58	MS	2iP+NAA+GA_3	RT(8)+vit B12	58.67[abcde]
M65	MS*	BAP+NAA+GA_3	RT(8)+vit B12	27.00[ef]
M6	½MS	-	-	8.67[f]
M60	½MS	-	Ad+Th	53.33[abcde]
M59	½MS	-	RT(8)+vit B12	39.00[cdef]

CD=40.35; Means in a column with same superscript are not significantly different from each other at 5% level.

NAA*= NAA concentration in M29, M53 and M62 was 0.1mg/l but the rest had 0.5mg/l.

BAP*= All the media with BAP had concentration of 1mg/l, except M32 that had 0.1mg/l and M29, M35, M53 and M62 that had 0.5mg/l.

The TDZ growth regulator contained in the media of preceding experiments was found promising for survival and multiple shoot initiation. Therefore, in Experiment 9(a) we investigated seven concentrations of TDZ to find out the optimum dose of this growth regulator. Mixed callus of the three accessions were cultured thrice at 2 weeks interval. The analysis of variance (Table 11) revealed non-significant mean squares for treatments (media) in both first and second subcultures indicating that there was no significant effect of different TDZ concentrations. However, the same treatments in the 3rd subculture were found significantly different, indicating that TDZ concentrations would have an effect on the callus

survival, higher concentrations giving higher survival rates (Table 12). Hence, the 3rd subculture was repeated and more detail observations were recorded at 2, 3 and 4 weeks interval for weight and size of callus tissues in addition to survival percentage (experiment 9b). However, this time the treatments were not significantly different for weight, size and survival at 2, 3 and 4 WAC (Table 13 & 14).

The percentage of callus survival varied from 50% to 86.67% in the 1st subculture, from 39.17% to 90.83% in the 2nd subculture and from 50% to 100% in the 3rd subculture. Media H15 with TDZ concentration of 10 mg/l gave consistently higher survival (78.95%) rates in all three subcultures. H14 (TDZ=7.5mg/l) showed 100% survival in the 3rd subculture and 78% in the 2nd subculture. Media H9 (TDZ 0.25mg/l) exhibited high survival (87% and 90%) in 1st and 2nd subculture, respectively but low (65%) survival in the 3rd subculture. The control (without TDZ) media showed the lowest survival in all subcultures as compared to those with different concentration of TDZ (Table 12).

The addition of TDZ to the media gave a generally a high percentage of callus, the response varying with time, regardless of the TDZ concentration (between 0.25 and 10mg/l), most callus having a pale green color. However, in M32 (previous best media used as a control) this response on callus survival was similar, but the callus had a deep green color, looking more vigorous than the ones in TDZ contained media.

Table 11. Analysis of variance for callus survival in different concentration of TDZ (Experiment 9a)

Source	1st Subculture		2nd Subculture		3rd Subculture	
	df	MSS	df	MSS	df	MSS
Rep	2	6069.48	3	598.60	1	50.003
Treatment	8	412.16	8	256.98	8	534.723**
Error	16	395.92	24	689.51	8	37.50
Total	26		35		17	

**Significant at 1% level of significance: Treatment: Media

Table 12. Effect of nine different concentration of TDZ on survival percentage of tissues sub-cultured thrice (Experiment 9a).

Media**	Growth Regulators(mg/l)	Mean survival (%) of tissue at two WAC done thrice		
		1st subculture	2nd subculture	3rd subculture
H8	-	50.00	39.17	50.00[d]
H9	TDZ(0.25)	86.67	90.83	65.00[c]
H10	TDZ(0.5)	77.78	66.67	95.00[a]
H11	TDZ(1.0)	61.11	44.05	80.00[b]
H12	TDZ(2.0)	61.11	80.36	90.00[ab]
H13	TDZ(5.0)	72.22	50.95	90.00[ab]
H14	TDZ(7.5)	77.78	68.75	100.00[a]
H15	TDZ(10)	77.78	81.85	95.00[a]
M32	BAP(0.1)	61.11	68.22	90.00[ab]
C.D.		ns	ns	14.1041

Means in a column with same superscript are not significantly different from each other at 5% level.

Table 13. Analysis of variance for callus survival in different concentration of TDZ (Experiment 9b).

Source	df	MSS		
		2nd Week	3rd Week	4th Week
Rep	1	88.89	45.34	1088.89
Treatment	8	450.00	554.08	555.56
Error	8	238.89	252.50	388.89
Total	17			

**Significant at 1% level of significance
*Significant at 5% level of significance
Treatment: Media

Table 14. Analysis of variance for callus size and weight in different concentrations of TDZ (Experiment 9b).

Source	df	MSS			
		Size			Weight
		2nd Week	3rd Week	4th Week	4th Week
Rep	1	33.62	35.84	10.27	0.011
Treatment	8	5.857	6.29	9.08	0.066
Error	8	3.855	6.50	8.90	0.098
Total	17				

**Significant at 1% level of significance
*Significant at 5% level of significance

Treatment: Media

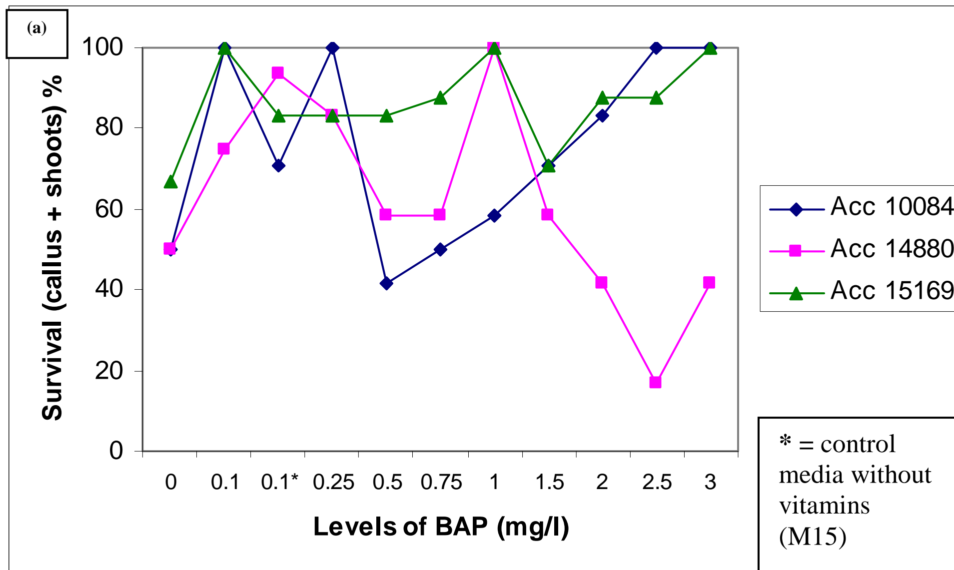
BAP seemed to be performing well in many of the previous experiments. So this experiment was designed to re-confirm what have been obtained so far and also to find out the optimum dose. So, we investigated ten concentrations of BAP. Experiment 13 has got all the three accessions. The first part of this experiment, (13a) was composed of both tissues (shoots as well as callus) grown from meristems and 13b had only callus remained from previous experiments. Analysis of variance for survival percentage at two WAC revealed significant differences amongst accessions, media, as well as a significant interaction between accession and media in both experiments 13a and b (Table 15). Therefore, results are presented in interaction graphs. In experiment 13a (Figure 10a), all the three accessions were doing well at a lower concentration of BAP (0.1, and 0.25 mg/l). Accessions 14880 and 10084 started to reduce survival at 0.5 and 0.75 mg/l of BAP while accession 15169 still showed good survival. At a higher concentration (beyond 1.5 mg/l), accession 14880 showed a drastic reduction on survival percentage of callus and shoots. In experiment 13b (Figure 10b), where only calli were evaluated, lower levels (0.1, 0.25 and 0.5 mg/l) of BAP were again good for all the three accessions. However, at a higher concentration (beyond 0.75 mg/l) accession 15169 had a lower survival of callus, while accessions 10084 and 14880 still maintained a relatively high survival. In experiment 13a, there were about half callus and shoots on accessions 14880 and 15169, whereas there were very few shoots for accession 10084. Figure 10c shows the percentage of shoots that were planted in each treatment. Lower levels of BAP were effective to maintain shoots alive while higher levels seemed to give lower survival, specially accession 15169 (Figure 10d), where some shoots were observed to turn into callus again. When we look at the percentage of shoots survived at the end of this experiment, lower levels (0, 0.1 and 0.25 mg/l) of BAP responded better than higher levels.

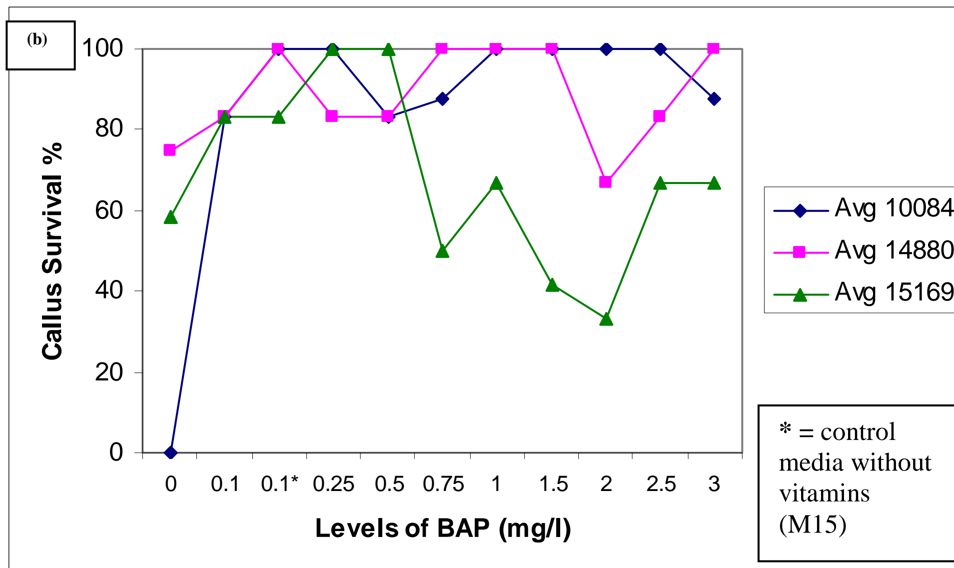
Table 15. Analysis of variance for survival percentage of cultured tissues (Experiment 13).

Source	df	MSS	
		Exp. 13a (callus+shoots)	Exp. 13b(callus only)
Rep	1	825.00	206.16
Media	10	1233.43**	858.99**
Accession	2	2681.02**	3666.02**
A*B	20	868.46**	773.49**
Error	32	162.996	251.805

Total	65

**Significant at 1% level of significance
WAC: Weeks after culture





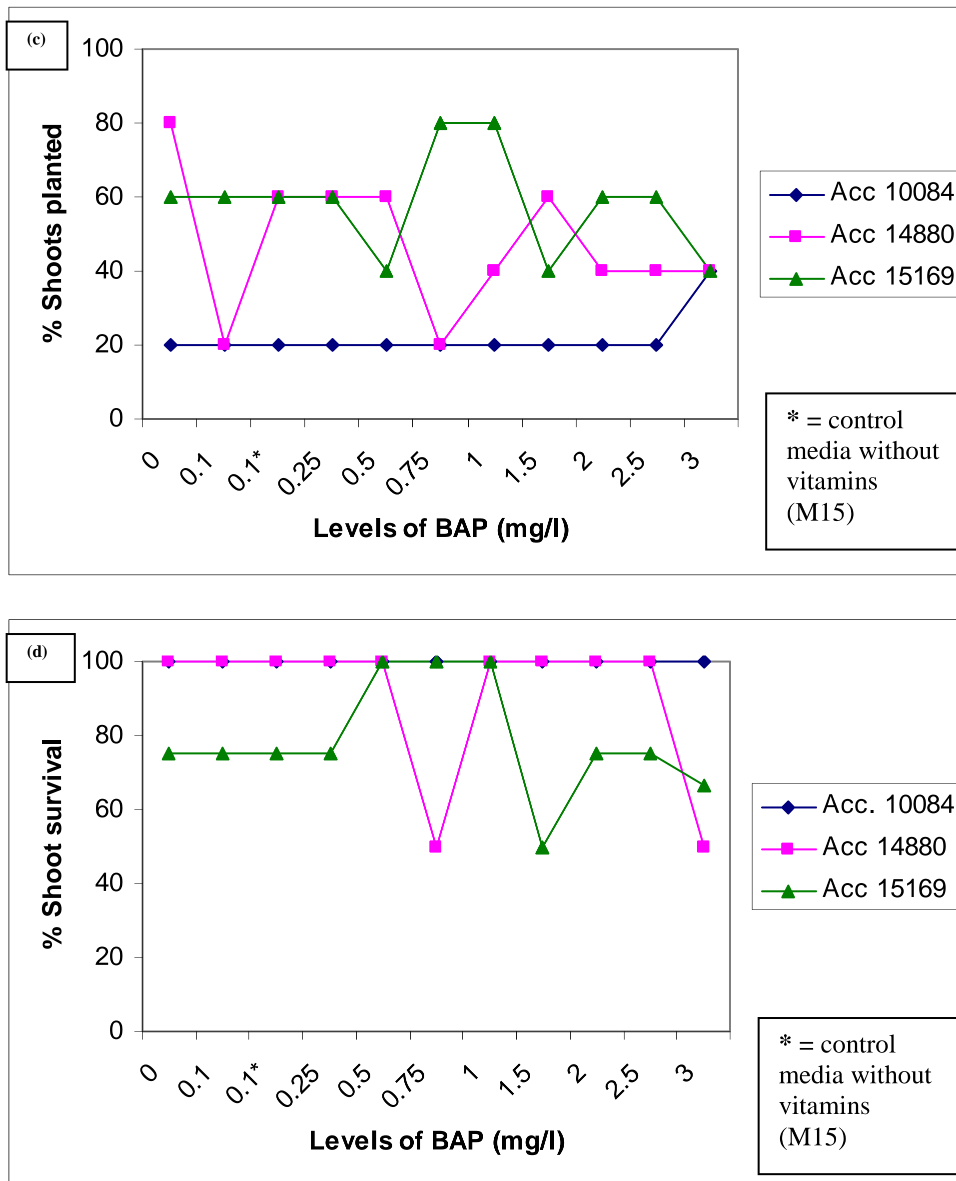


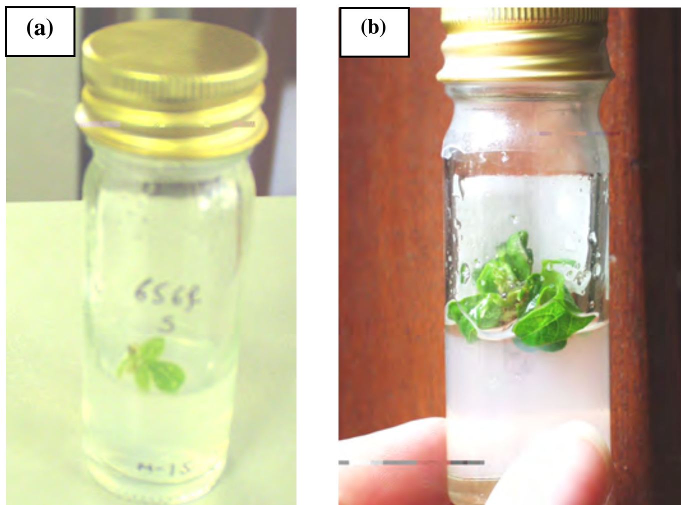
Figure 10. Effect of different levels of BAP on survival percentages of shoots and callus and percentage of shoots maintained in these media for the different accessions (a) Survival percentage of callus + shoots – Experiment 13a (b) Survival percentages of callus – Experiment 13b (c) Percentage of shoots cultured – Experiment 13a (d) Percentage of shoots maintained after 2 weeks of culture – Experiment 13a.

4.3. Induction of roots

A few drops of liquid rooting media containing ½MS and an auxin IBA (1 mg/l) were poured on top of the solid M15 media with the large shoot cultures obtained after completion of

Experiment 13a. No rooting was observed in the beginning, however, plants grew vigorously with large and bright green leaves. Due to the large size of the plants, larger containers were then adopted (40 ml jars with wide mouth) and shoots were subcultured again into M15 and the liquid rooting media, with a stronger concentration of IBA (5 mg/l) and ¼ G-B5 + 7.5 mg/l sucrose was poured again on top. Initially we had 44 (22%) shoots from a total of 197 plants in 13a. However, we were left with 24 (12%) shoots at the end of the experiment. These shoots were subcultured into the same media again and multiple shoot initiation was observed especially at lower concentrations (0, 0.1 and 0.25 mg/l) of BAP (Figures 11a and b). After 1 week of culture in the rooting media, roots started to appear at the base of the elongated shoots (Figure 11c) in 8 plants of a total of 14 plants (Table 16), specially at lower levels of BAP (0, 0.1 and 0.25 mg/l). However, possible due to the type of the containers (that had loose caps) as well as the time of the year (during the rainy season there is a much higher rate of contamination in the tissue culture environment) there was a high contamination of fungi and bacteria and many of these large, vigorous and rooted plants did not survive time enough to be acclimatized in the greenhouse.

Roots were also later on observed in a few callus cultures kept for longer period of time after the completion of the experiments, though there were no shoots produced on this callus (Figure 11d).



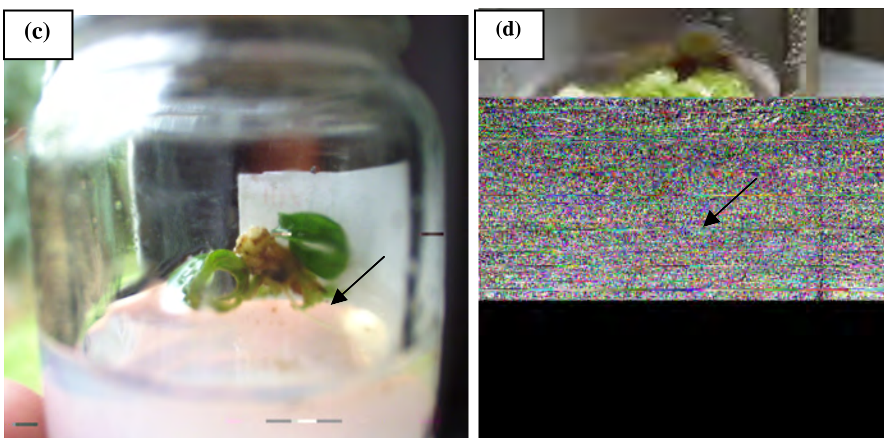


Figure 11. (a and b) Elongated shoots with leaves on BAP containing media (M15) at 2 and 4 WAC. **(c)** Root induction at the base of elongated shoot (d) Root formed on callus after long period of culturing (experiment 13).

Table 16. Effect of different concentrations of BAP on rooting after 1 week of culture in rooting media (Subculture of Experiment 13a).

BAP (mg/l)	Number (Percentage) of plants with roots		
	10084	14880	15169
0	0	0	0
0.1	0	2(40%)	2(67%)
0.1*	1(50%)	2(67%)	0
0.25	0	1(33%)	0
0.5	0	0	0
0.75	0	0	0
1.0	0	0	0
1.5	0	0	0
2.0	0	0	0
2.5	0	0	0
3.0	0	0	0

0.1* - Control media without vitamins (M15)

5. DISCUSSION

The eradication of virus on infected *Mucuna pruriens* plants is highly important. An antiviral treatment capable of eradicating virus from infected plants is either not available or considered environment unfriendly. Thermotherapy, combined with meristem culture, is a standard procedure for virus elimination. However, *in vitro* regeneration and establishment is often difficult, poorly repeatable and less efficient (Veltcheva *et al*., 2005; Herselman and Mienie, 1995; and Grum *et al.,* 1998). *Mucuna pruriens* is known to produce phenolic compounds that have interfered with our effort in producing tissue-cultured plantlets. Often both shoot and callus tissue cultures turned brown/black a few weeks after culture, resulting in loss of cultures, possibly due to an increase in phenolics. This has also been observed in *Vicia faba* (Khalafalla and Hattori, 1999).

According to Jayanand *et al.* (2003), shoot development as a result of proliferation of pre-existing meristems is not reproducible and it needs optimization of various tissue culture variables such as different nutrients and their combination. The size of meristems has an impact on establishment of cultures. Larger meristems grow faster before the stimulus that induces callus formation. However, they are not as efficient as smaller ones in eliminating viruses (Juan *et al.,* 2004). In our studies, smaller meristems were used since our aim was the development of virus-free plants. Direct organogenesis or shoot regeneration through a callus phase are the usual routes of *in-vitro* plant production. Direct organogenesis is often desirable in maintenance of genetic stability of elite clones.

5.1. BAP effect

Cytokinins are important for shoot regeneration. The concentration of cytokinins is also known to be a critical factor for shoot organogenesis. Previous reports (Kartha *et al.,* 1981; Benedicic *et al.,* 1997; Veltcheva *et al.,* 2005; Malik *et al.,* 2005) indicated that BAP has been used as a principal cytokinin for induction of multiple shoot buds in soybean, cowpea, peanut, chickpea, bean and *Garcinia indica*. However, an increase in BAP concentration beyond the optimal level led to decrease in shoot length and aggregations of shoot buds. In our case, BAP (0.1mg/l) supplemented medium was found to be the most effective one for shoot initiation and elongation (M29, M35, M15 and M32, Table 1). Particularly, Experiment

13a (Table 15; Figures 10a, c, d & 11) revealed that it is possible to maintain and elongate shoots in M15 for several weeks. Other authors also found BAP to be effective for shoot formation, although at a lower level (for different species). Faisal, *et. al.* (2006) indicated that an optimum concentration (5.0 µm) of BA has been found more effective in shoot formation. According to Zaerr and Mapes (1982), factors that contribute for the success of BAP may be either its ability to easily get metabolized in plant tissues or its ability to induce other natural hormones within the plant tissue.

5.2. TDZ effect

Next to BAP, TDZ, a substituted phenyl urea, exhibiting a strong cytokinin-like activity (Mok *et al.,* 1982) has been reported to induce adventitious shoot buds in a number of plant species including *Capsicum* species (Peddaboina *et al.,* 2006) and *Linum* species (Mundhara and Rashid, 2006). However, these shoots were often difficult to root. TDZ has disadvantages such as inducing a stunted growth of shoots, abnormal leaf morphology, and 'carryover effect' creating difficulty in tissue-cultured shoots to root. Similarly, we also obtained good shoot growth (Experiments 4&6 and Table 8) when adding TDZ to our growth media and we were encouraged in the beginning to see elongated shoots in such media (Figure 9). Therefore, we also further did an experiment with different concentrations of TDZ using only callus tissues (Experiment 9). The callus grew big, however, no organogenesis from callus was observed (Table 12), neither any rooting. We faced a similar problem of inducing roots in tissue cultured shoots grown in TDZ containing media (Experiments 11&12) as is referred somewhere. Bohmer *et. al.* (1995) suggested to use lowest but effective TDZ concentration and to keep the culture on TDZ containing media for the least duration that is specific for each species. Peddaboina *et al.* (2006) suggested transferring cultures into BAP plus an auxin (IAA) containing media for maximum shoot elongation.

5.3. Genotype effect

Genotypic differences among the three accessions in survival percentage as well as shoot formation (Table 3&4, 5&6, 15 and Figure 10) were observed in experiments 1, 2 and 13. The accession 14880 was the best followed by 15169 and the least was 10084 in the 1st

experiment (Table 4). In the series of experiments carried out, we were in short supply of culturing materials of accession 10084, due to its poor consecutive survival. So we were forced to rely on the rest two accessions (14880 and 15169), to carry out the remaining experiments planned, especially using 14880 that had more surviving shoots and callus in general. In the 2nd experiment, 15169 survived better than 14880, but here only callus tissues were compared. Then in the last experiment, 13a, there was a strong interaction between accessions and media. 14880 seemed to be more sensitive to the higher levels of BAP (Figures 10a and 10d). In general, though we observed inconsistency in the response of these accessions in respect to callus growth, better response in shoots was observed consistently in accession 14880. So we can say that accession 14880 was the best in shoot formation. Genotypic effect is well established in tissue culture response. Different genotypes had different physiological requirement of plant growth regulators for *in vitro* shoot regeneration (Malik *et al.,* 2005).

A differential response in callus induction and survival was noticed when callus were cultured in different media supplemented with different cytokinins alone or along with auxins at different concentrations. However, the callus remained undifferentiated into shoots regardless of the media and growth regulators used.

It was also observed along these experiments, that there was a distinct difference between the response of the various tissues, i.e., the callus response to the media treatments was often different from the shoot response to the same treatments. This shows physiologic variation of responses on the tissues.

5.4. Rooting plants

Difficulty in rooting of shoots induced in TDZ and BAP containing media was noted. Fratini and Ruiz (2002) reported the inhibitory effect of both TDZ and BAP on root formation in lentil by inducing root swelling, stunting and callusing if higher concentration is used. Some researchers suggested to repeatedly subculture regenerated shoots into low concentration of cytokinin in each step. Others recommended the reduction on the exposure time to cytokinins; others, to reduce the salt in the shooting media and transfer to rooting media. In the last experiment, successful rooting has been observed in shoots grown in low levels of

BAP containing media when transferred into media with low salts, low sugar and low cytokinin supplemented with an auxin IBA. Similar results have been observed in faba bean when transferred into low salt containing media (Khalafalla and Hattori, 1999). There were lots of calli remained after the completion of all the experiments and we observed few roots on those calli after culturing the callus on several different media for longer periods of time. It is difficult to tell which medium or growth regulator triggered root induction on those calli since they have been cultured in several of them.

Chattopadhyay *et. al.* (1995) for the first time reported successful micro-propagation in *Mucuna pruriens* using seedling explants particularly epicotyls and hypocotyls + cotyledon. They observed multiple shoot regeneration after an initial callus phase on RT medium supplemented with 2.7 µm NAA and 9.8 µm 2-iP. Although explants used in the present work were meristems, which differed from those referred in this article, yet the media composition used for shoot induction was the same as used by these workers. However, it did not show any better positive response in our case (RT vitamins in Experiments 2, 3 & 4).

6. CONCLUSIONS

In our studies we have demonstrated and standardized the culture media responsive for elongation and production of multiple shoots and roots at the base of elongated shoots. After a long series of experiments we could conclude that *Mucuna pruriens* grows better in:

- Media with G-B5 salts.
- BAP between 0.1 and 0.25 mg/l as a growth regulating hormone.
- TDZ at 0.25 and 0.5 mg/l improved shoot elongation only for the first few weeks of growth.
- IBA (5 mg/l) did induce rooting in low concentration salts and sucrose in a few plants. However, further investigations are required in order to enhance the efficiency to induce rooting in elongated shoots.
- The inclusion of vitamins (Ad and Th) did induce initial shoot formation from meristem, but later on media without vitamins was better to maintain shoots alive and green for a longer period.
- There was a strong response in both genotypes and types of tissues. Specific media adjustments could be further done to obtain the best growth in each genotype. In addition, results obtained from callus cultures cannot be directly adapted or compared with shoots.

7. RECOMMENDATION

Due to the long duration of this type of experiments and the limited time frame of the student grant, we can not proceed any further. However, this work was continued thereafter by ILRI, where studies continued to obtain large numbers of rooted plants based on these results. Therefore, future work should proceed to:

- promote more roots on the developed shoots
- to acclimatize and transfer the in vitro developed plantlets into pots
- further investigate roots on callus

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