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Novel Plant Regeneration for Egyptian Sweetpotato (*Ipomoea Batatas* (L.) Lam.) Abees Cultivar *via* Indirect Organogenesis Stimulated by Initiation Medium and Cytokinin Effects

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Abstract: In this study, a simple and efficient plant regeneration system in Egyptian sweetpotato Abees recalcitrant cultivar *via* indirect organogenesis was established. Two initiation medium and different regeneration medium were tested. Hormone free initiation medium (HFIM) treatment was found to be critical for induction of regenerative callus. Likewise, callus diagnostic structural histology confirmed *de novo* differentiation of meristematic domes in induced callus tissues and their further development into bud primordia. Shoot regeneration was dramatically improved by subculture of the initiated callus from HFIM onto different enriched cytokinin regeneration medium. Although, an overall analysis of variation also revealed a significant response for media used for shoot regeneration. The highest significant number of regenerated shoots per shooted callus (2.33) and frequency of regenerated shoots (80.90%) were obtained on benzyl adenine containing medium (BARM). Subsequently, regenerated shoots were rooted on hormone free medium (RM). Regenerated plants were acclimatized in controlled environment growth chamber and successfully established in the greenhouse. Hence, we report a reliable regeneration system for local recalcitrant cultivar Abees which could be used to exert selection pressure to abiotic stress or to transfer genes of agronomic interest and produce transgenic plants.

Key words: growth regulators, callogenesis, adventitious buds, *de novo* meristem formation, organogenesis, regeneration.

INTRODUCTION

Sweetpotato (*Ipomoea batatas* (L.) Lam.) is a gamopetalous dicot and belongs to the order *Polemoniales* and the family *Convolvulaceae*. Sweetpotato ranks as the world's seventh most important crop after wheat (*Triticum aestivum*), rice (*Oryza sativa*), maize (*Zea mays*), potato (*Solanum tuberosum*), barley (*Hordeum vulgare*), and cassava (*Manihot esculenta* Crantz.) (Hironori *et al.,* 2007). Sweetpotato is a tuber-bearing species and represents an economically important crop in tropical, subtropical and warm temperate regions (Sihachakr *et al.,* 1997). Luo *et al.,* 2006 stated that sweetpotato is grown in more than 100 countries with the world's average of 15 tons per hectare. In Egypt, the sweetpotato variety "Abees" a purple skin with orange-flesh, is rich in β-carotene, and presents approximately 60% of the total sweetpotato cultivated area to 30,000 acres (FAO.org, 2007). Also, Sweetpotato has received increased attention because it can adapt to a wide range of environmental conditions and grow on marginal areas with poor soils of limited fertility and inadequate moisture (Bioethics, 2004). Sweetpotato a good starch source, in addition fresh sweetpotatoes provide about 50% more calories than white potatoes (Purseglove, 1968) and is a valuable source of food and animal feed (Yujun *et al.,* 2007). Moreover, it is expected to be used in immense quantity as raw materials for biodegradable plastics and for bio-fuel of automobiles (Kozai *et al.,* 1996a; 1996b). Furthermore, sweetpotato have a high production yield of biomass, thus it could have large impact as industrial material for application in biotechnology as a source for medicinal applications (Thomas *et al.,* 2005). In recent years, the improvement of sweetpotato is vital as the productivity is adversely affected by abiotic stresses, insect pests and fungal and viral infections The greatest danger has been identified as the susceptibility of sweetpotato to virus diseases which has caused substantial yield reduction of up to 80% (Abo El-Abbas *et al*., 1998; Karyeija

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et al., 2000; Odame *et al.,* 2002). Moreover, conventional breeding efforts and Genetic improvement have been seriously limited by difficulties in sexual crosses, mainly due to incompatibility and male sterility within species (Martin, 1970) hexaploid nature, dichogamy, seed dormancy, abnormal seed and seedling development (Sihachakr and Ducreux, 1987), as well as, specific physiological requirements for flowering (Martin and Jones, 1971).

Tissue culture techniques have opened a new frontier in agricultural science by addressing food security and agricultural production issues (Oggema *et al.,* 2007b) and led to more emphasis on the use of biotechnological methods for genetic improvement of exacting traits in sweetpotato. Little work has been achieved, particularly the exploitation of somaclonal variation (Sihachakr *et al.,* 1997) and genetic transformation (Otani, *et al.,* 2003). Since, the application of new techniques for improvement of sweetpotato crops, mostly including the exploitation of gene transfer by genetic transformation and somatic hybridization, requires the control of plant regeneration from tissue cultures (Sihachakr *et al.,* 1997). Therefore, development of a reliable *in vitro* plant regeneration procedure for a cultivar is a prerequisite for its improvement by creating genetic variability *via* biotechnological methods involving direct gene transfer (Sihachakr *et al.,* 1997). As success of any transformation strategy depends largely upon the regeneration capability of the target explant (Chugh and Khurana, 2003). Thus, the lack of efficient system for regeneration has been a bottleneck for the application of biotechnology in sweetpotato (Liu *et al.,* 2001). Although, one of the main difficulties is the control of plant regeneration, for which sweetpotato is considered a recalcitrant species (Sihachakr and Ducreux, 1993). In most cases evidence for plant regeneration in sweetpotato were restricted to few genotypes, and when attempts were made to extend this into a wide range of genotypes, the majority were found to be recalcitrant (Liu *et al.,* 2001), additionally, sweetpotato is recalcitrant to regeneration because each cultivar shows different response *in vitro* (González *et al.,* 1999) since every cultivar varies widely in its response to plant regeneration (Gosukondan *et al.,* 1995). For these reason, genotype is one of the most important factors affecting the evolution of the *in vitro* culture, regeneration (Jarret and Gawel, 1991) and consequently transformation response.

In the beginnings, Cavalcante *et al.* (1994) produced plantlets from lateral bud derived callus of sweetpotato onto initiation regeneration medium supplemented with 2,4-D and BA. Then, Newell *et al.* (1995) obtained best callus induction and best shoot regeneration was onto medium containing NAA and BA after a series of plant growth regulator changes over 6 months. Subsequently, Otani and Shimada (1996) recorded that picloram in initiation medium was most suitable from eight kinds of auxins tested for further sweetpotato shoot regeneration from shoot apices explant. Moreover, Zheng *et al.* (1996) recorded that rapid and repetitive plant regeneration was in only one genotype of sweetpotato i.e. PI318846-3 onto media devoid of hormones after using firstly an initiation medium supplemented with 2,4-D. Then, Al-Mazrooei *et al.* (1997) found that type of auxin used in initiation medium is not only very critical for shoot regeneration which was obtained afterward onto medium without growth regulators, but also is specific to the individual cultivars. Also, Sihachakr *et al.* (1997) cultured lateral buds onto initiation medium containing 2,4-D to initiate callus then maintained onto medium containing 2,4-D and BA, after that regenerated shoots onto regeneration medium free from hormones. Moreover, shoots were regenerated from callus initiated and maintained onto medium containing 2,4-D and subcultured onto regeneration medium containing ABA (Liu *et al.* 1997, Dhir *et al.* 1999 and Liu *et al.* 2001). On contrary, shoot regeneration from petiole derived calli of sweetpotato cv. Genki was produced using regeneration medium containing BA only. (Wang *et al.* 1999). On other hand, plants were regenerated *via* organogenesis onto enriched auxin cytokinin medium containing NAA and BA (Morán *et al.* 1998 and Gong *et al.* 2001). On contrary, Song *et al.* (2004) and Thomas *et al.* (2005) produced callus of sweetpotato plants onto initiation medium containing 4-FA and regenerated shoots onto medium contained ABA and GA_3. Subsequently, shoot regeneration procedure was achieved *via* organogenesis from stem explants onto medium supplemented with NAA only (Gong *et al.*, 2005). Lately, Luo *et al.* (2006) achieved two-step organogenesis regeneration using a two-hormone protocol in which the explants were first kept on an initiation medium containing 4-FA and then transferred to a regeneration medium containing zeatin and they reported that initial short callus inducing step on 4-FA medium in the two-hormone protocol seems to be needed. Recently, Gibum *et al.* (2007) produced plants from apical meristem derived callus initiated onto medium supplemented with 2,4-D and regenerated onto plant regeneration medium containing BA. Furthermore, Oggema *et al.* (2007a) regenerated shoots from leaf derived callus initiated onto medium containing 2,4-D and BA or BA alone and regeneration medium containing ABA and BA. Also, Triqui *et al.* 2007 induced lateral buds from callus when cultured onto initiation medium supplemented with various concentrations of auxins i.e. 2,4-D or 2,4,5-T or picloram, whereas, 2,4,5-T or picloram were highly significant and obtained shoots when cultured onto regeneration medium either hormone-free or supplemented with combination of growth regulators.

In order to improve *via* biotechnology applications our Abees cultivar, the major sweetpotato cultivar in Egypt, it is crucial to establish an *in vitro* regeneration system which in turn will also open the door for the applications of transformation technologies. In the present study, an efficient regeneration system *via* organogenesis for cv. Abees was established using the stimulating effects of the interaction between two callus initiation media and five regeneration media. It was clearly proved that the short callus inducing step onto hormone free initiation medium followed by subculturing onto enriched cytokinin regeneration medium is strongly needed for successful plant regeneration in our local cultivar Abees.

MATERIALS AND METHODS

Plant Material and Explant:

Abees, which is one of the most important orange-fleshed sweetpotato cultivars (*Ipomoea batatas* (L.) Lam. in Egypt was used as plant material source. Virus-free plant material was micro-propagated *in vitro* using nodal cutting system as described by Mervat 2007. Cultures were routinely subcultured every 6 weeks onto a fresh MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and maintained at 28ºC under cool white fluorescent light 300 foot-candle for 16 h /8 h light/dark cycle. Dissected stem segments not including buds from the *in vitro* manipulated stock were used as an explant.

Callus Initiation:

For callus initiation, explants were cultured onto two different callus initiation media to evaluate their effects on different regeneration efficiency parameters i.e. shooted callus percentage (number of shooted callus/number initiated callus x 100), number of regenerated shoots per shooted callus (number of regenerated shoots/number shooted callus), regenerated shoots frequency (shooted callus percentage x number of regenerated shoots per shooted callus) and rooted callus percentage (number of rooted callus/number initiated callus x 100). The two tested media basically contains Murashige and Skoog salts (Duccifa Co., Netherlands) supplemented by 100 mg/l myo-inositol, 30 g sucrose, 2.2 g Phytagel as a solidifying agent and pH at 5.7. The first callus induction medium (PIM) contains 0.2 mg/l picloram as an auxin source; on the contrary, the other medium (HFIM) was free from auxin source. Cultures were then incubated in dark for two weeks in controlled growth chamber (Shel-Lab, USA) at temperature of 28°C. Subsequently cultures were transferred to a growth chamber under cool white fluorescent light 300 foot-candle for 16 h /8 h light/dark cycle for one more week.

Plant Regeneration:

Five different regeneration media had been used to evaluate their effects on different regeneration efficiency parameters; previously mentioned. Induced three weeks-old calli were subcultured onto glass jars containing 50 ml of MS medium (MS salts and vitamins, 30 g/l sucrose and 2.2 g/l Phytagel) supplemented with different growth regulators i.e. 10 mg/l benzyl adenine (BARM) or 4 mg/l zeatin riboside (ZRRM) or 0.1 mg/l thidiazuron (TDZRM) or 0.2 mg/l picloram (PRM) or free from growth regulators (HFRM) as a control. Subcultured callus were kept in the growth chamber under the same light and environmental conditions as previously stated for three weeks and subcultured once more for more three weeks.

Anatomical Analysis:

For the anatomical analysis, callus culture samples were fixed in FAA (formalin, glacial acetic acid and 50% ethyl alcohol), dehydrated through an ethanol series, embedded in paraffin, sectioned at 10-12 µm thick by rotary microtome (Reichert 820, USA), stained in safranin-fast green combination and mounted in Canada-Balsam (Johansen, 1940). All sections were examined with Microscope (AO Scientific Instrument, Illuminator Model 1135, USA), photomicrographs were taken by digital camera Kodak Easy Share cx6330.

Rooting and Acclimatization:

Regenerated shoots, 1 cm or longer, were cultured singly in glass jar containing 50 ml of MS medium supplemented with 20 g/l sucrose free from growth regulators (RM). Obtained plantlets were then transferred into pots in controlled growth chamber incubator and successfully established in the greenhouse.

Statistical Analysis:

Data obtained was recorded after nine weeks and were exposed to the proper statistical analysis of complete randomized design (Snedecor and Cochran, 1969) in three replicates. Means obtained were differentiated using Duncan's new multiple range test as described by (Duncan, 1955). To improve the normality of data, transformations were used as suggested by (Anscombe, 1948).

RESULTS AND DISCUSSION

Callus Initiation and Plant Regeneration:

Establishment of regeneration system in sweetpotato is of potential importance to sweetpotato quality improvement; however, successful transformation cannot be achieved unless efficient plant regeneration to be established (Gong *et al.,* 2005). Numerous protocols reporting sweetpotato regeneration were examined and failed to regenerate shoots for our cultivar Abees. The aim of this work was to establish an efficient regeneration system for the recalcitrant cultivar Abees using the stimulating effects of the interaction between two tested callus initiation media and five regeneration media.

I. Initiation Medium Effects:

Significant effects were scored in plant regeneration as affected by the two tested initiation medium i.e. picloram initiation medium (PIM) and hormone free initiation medium (HFIM) and differences reached the 5% level of significance as shown as in Table (1). HFIM showed superiority and proved to be the favorable medium that acts as complementary factor with endogenous hormones for production of the highest shoot regeneration characteristics i.e. shooted callus percentage, number of regenerated shoots per shooted callus and frequency of regenerated shoots; whilst PIM was more effective for root regeneration (rooted callus percentage) as shown in Table (1). Results obtained could be suggested that sensitivity to growth hormones addition is probably affected by the endogenous hormonal balance in cells and plant regeneration interactions, which control many circumstances expressed by cells. Limited data had been reported on the influence of initiation medium in sweetpotato regeneration. The type of auxin used in initiation medium was found significant for plant regeneration and specific to the individual cultivars (Al-Mazrooei *et al.,* 1997). González *et al.* (1999) found that influence of auxin enriched initiation medium was observed to be greater than that with cytokinin enriched medium to induce organogenesis. Chee *et al.* (1990), Newell *et al.* (1995), Dhir *et al.* (1998), Lawton *et al.* (2000) and Gong *et al.,* (2001) maintained sweetpotato explants onto auxin cytokinin enriched medium as a callus initiation medium. Although, Otani and Shimada (1996), Al-Mazrooei *et al.* (1997) and Triqui *et al.,* (2007) used initiation medium supplemented with picloram as an auxin source to induce callus from sweetpotato explants and stated that it was critical for further plant regeneration. Luo *et al.* (2006) recorded that initial short callus inducing step on auxin containing medium (4-FA) seems to be needed. On the other hand, Oggema *et al.* (2007a) synchronized with our results, pointed out that the lower 2,4-D concentration in initiation medium resulted higher shoots regeneration and lower regenerated roots. In preliminary experiments, it was noticed that HFIM and PIM were favorable to induce callus for Abees cultivar; respectively (data not shown). Most likely, in the present investigation, maintaining of Abees explant for 3 weeks onto HFIM (free from growth regulators) proved to be the appropriate medium not only for callus induction but for the promotion of cell differentiation and further plant regeneration. It could be suggested that sensitivity to growth hormones addition is probably affected by the endogenous levels of hormones in cells and it is reasonable to assume that differences in response *via* different sweetpotato cultivars in literature cited, resulted from the genetically differences among genotypes.

II. Regeneration Medium Effects:

In regeneration medium, plant growth regulators play a pivotal role in the regulation of morphogenesis; both exogenous and endogenous growth regulators affected cell culture and manipulate its performance. Initiation of morphogenesis among genera, species, and even cultivars varied with type and concentration of required auxins and cytokinins. In Table (2), enriched cytokinin regeneration medium BARM scored a significant increase in shooted callus percentage, number of regenerated shoots per shooted callus and frequency of regenerated shoots, while PRM scored the lowest values. These results may be regarded to that benzyl adenine medium probably has a significant assignment in increasing plant regeneration as it increases cell potency towards differentiation. Similar results were reported by Wang *et al.*, (1999) and Gibum *et al.,* (2007) whom produced shoot regeneration in sweetpotato onto regeneration medium containing BA. On other hand, Gosukonda *et al.,* (1995) induced adventitious shoot regeneration in sweetpotato using regeneration medium containing thidiazuron. On contrary, hormone free medium has an opposite manner; it scored the highest significant value in rooted callus percentage. Probably, it could be attributed to endogenous hormone and its excessive polarity to induce callus and root regeneration which in turn decreased cell potency towards shoot differentiation. In fact, endogenous growth regulators levels within plant explants, with the interaction between exogenous hormones probably influence regeneration response. Synchronized results were obtained by González *et al.* (1999) whom obtained root formation from CEMSA-78354 cultivar on hormone free medium but with very low frequency and indicated that it may due to the endogenous hormonal levels of tested cultivar.

Table 1: Effect of Initiation medium on plant regeneration characteristics.

Initiation medium	Shooted callus percentage	Number of regenerated shoots per shooted callus	Frequency of regenerated shoots	Rooted callus percentage
PIM	0.89 B	0.13 B	0.59 B	31.11 A
HFIM	18.94 A	1.09 A	31.00 A	17.56 B

-Means followed by different capital letters in columns are significantly different at P = 0.05 according to Duncan's multiple range test.

Table 2: Effect of regeneration medium on plant regeneration characteristics.

Regeneration medium	Shooted callus percentage	Number of regenerated shoots per shooted callus	Frequency of regenerated shoots	Rooted callus percentage
HFRM	2.22 C	0.33 D	1.48 C	51.67 A
BARM	17.36 A	1.17 A	40.45 A	23.33 B
ZRRM	18.89 A	0.50 C	18.89 B	16.67 B
TDZRM	10.00 B	0.89 B	17.78 B	12.22 C
PRM	1.11 D	0.17 E	0.37 C	17.78 B

- Means followed by different capital letters in columns are significantly different at P = 0.05 according to Duncan's multiple range test.

III. Initiation Medium × Regeneration Medium Interaction Effects

One of the most important approaches for overcoming *in vitro* recalcitrance problems is the optimization of plant growth regulators; however, cultures are under the control of both endogenous and exogenous plant growth regulation, therefore, achieving the correct exogenous balance of the key hormones auxin and cytokinin can be critical, also, their appropriate application can effectively overcome certain recalcitrance problems (Erica, 2000). Effect of interactions between initiation medium and regeneration medium on plant regeneration characteristics were presented in table (3) and fig. (1) whereas, shooted callus percentage, number of regenerated shoots per shooted callus and frequency of regenerated shoots were affected significantly at different tested regeneration medium i.e. HFRM, BARM, ZRRM, TDZRM and PRM among the two tested initiation medium (PIM and HFIM) and were high enough to reach the 5% level of significance. Generally, enriched cytokinin regeneration medium BARM under HFIM treatment (hormone free initiation medium) scored the highest values of plant regeneration in term of number of regenerated shoots/shooted callus or/and frequency of regenerated shoots, with the exception, the highest values of shooted callus percentage were scored by ZRRM with slight insignificant increase than BARM. On other hand, no shoot regeneration was produced either onto BARM, ZRRM, TDZRM and PRM under PIM treatment or onto HFRM under HMIF treatment. In contrast, HFRM under PIM treatment scored highest significant values of rooted callus percentage, while the lowest values under HMIF treatment were recorded onto TDZRM, while BARM produced no roots. It be concluded that this consequences is probably due the presence of different *in situ* endogenous hormonal levels on the cellular level, which probably affect the cell potency manner towards shoot and root differentiation. There are few reports of *in vitro* regeneration of sweetpotato plants *via* organogenesis (Porobodessai *et al.,* 1995; Gosukonda *et al.,* 1995; González *et al.,* 1999; Gong *et al.,* 2001; Gong *et al.,* 2005 and Luo *et al.,* 2006). Several authors drew the attention to the effect of initiation and regeneration medium on stimulation of plant regeneration for sweetpotato as Al-Mazrooei *et al.* (1997) whom obtained shoot regeneration onto medium without growth regulators and pointed that auxin initiation medium is critical and specific to the individual cultivars. Moreover, two-step sweetpotato organogenesis regeneration was established by Luo *et al.* (2006) using firstly initiation media containing 4-FA and finally zeatin in regeneration media and they indicated that the initial short callus inducing step in the two-hormone protocol seems to be needed. Also, Gibum *et al.* (2007) initiated callus from apical meristem onto medium supplemented with 2,4-D and regenerated shoots onto BA regeneration medium. In contrary, Oggema *et al.* (2007a)` synchronized with our results regenerated shoots from callus initiated on medium containing three different levels of 2,4-D (zero or 0.5 or 1 mg/l) and BA and regeneration medium containing ABA and BA, and observed that the lower the 2,4-D concentration in maintaining medium resulted higher shoots regeneration and lower regenerated roots.

Table 3: Effect of interactions between initiation medium and regeneration medium on plant regeneration characteristics.

Initiation medium	Regeneration medium	Shooted callus percentage	Number of regenerated shoots per shooted callus	Frequency of regenerated shoots	Rooted callus percentage
PIM	HFRM	4.45 C	0.67 D	2.97 C	53.33 A
	BARM	0.00 E	0.00 F	0.00 D	46.67 B
	ZRRM	0.00 E	0.00 F	0.00 D	22.22 C
	TDZRM	0.00 E	0.00 F	0.00 D	20.00 C
	PRM	0.00 E	0.00 F	0.00 D	13.33 D
HFIM	HFRM	0.00 E	0.00 F	0.00 D	50.00 AB
	BARM	34.72 A	2.33 A	80.90 A	0.00 F
	ZRRM	37.78 A	1.00 C	37.78 B	11.11 D
	TDZRM	20.00 B	1.78 B	35.56 B	4.45 E
	PRM	2.22 D	0.33 E	0.74 D	22.22 C

- Means followed by different capital letters in columns are significantly different at P = 0.05 according to Duncan's multiple range test.

Fig. 1: a. Abees cultivar calli initiated onto initiation medium (HFIM) showing dark green meristematic domes (arrows). b. Abees calli showing organogenesis (arrow) onto regeneration medium (BARM). c. Two primordial shoot initiation (arrows) onto BARM. d. Calli showing multiple primordial shoot regeneration. e. Multiple shoot regeneration on BARM showing three shoots and multiple primordial shoots (arrow) formed on one single explant. f. Whole rooted plantlets grown onto rooting medium (RM). g. Well root formation from Abees regenerated shoots on rooting medium (RM). h. Acclimatized plantlet into pot in controlled growth chamber incubator. i. Successfully established plants in greenhouse.

Anatomical Analysis:

Four weeks after explant culturing, growing initiated calli begun to release compact dark green opaque cells. Histological sections showed that they were meristematic structures (fig.2). These structures actually then developed, releasing the apical dome meristem cells and subsequently possess well-defined leaf primordia. It was observed presence of callus development definitely affected by the exogenous growth regulators i.e. benzyl adenine, zeatin ribozide, thidiazuron and picloram, whereas, anatomical sections of these developing calli showed that they were able to induce a primary callus consisting meristem cells and tended to release high cyto-plasmic cells, which appeared to rapidly form differentiated cell masses. It seems that subculture of imitated callus on regeneration media, provoked a callogenic reactivation to form meristematic cells. High nucleo-plasmic ratio and dense cytoplasm, exhibited by these cells are essential characters to obtain a high differentiation frequency. Anatomical observations have shown buds regeneration, these differentiated meristematic structures observed were likely derived from the meristematic cells that differentiated after the subculturing onto regeneration media. Organization and loose of parenchymatous tissue, consequently organized the *de novo* bud primordial structures of large vacuolated and differentiated cells with a high nucleo-plasmic ratio, conspicuous nucleus and nucleoli, and which thereby look like those of shoot primordia. Histological studies revealed *de novo* formation of meristematic centers in callus and their further development into bud primordia and exhibiting the formation of meristemoids shoots.

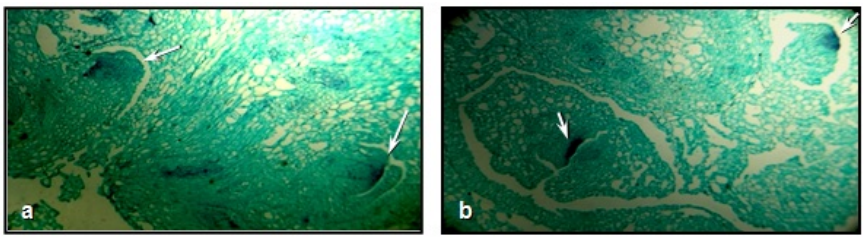

Fig. 2: Sweetpotato callus cultured onto regeneration medium showing shoot-bud primordia (arrows)
(a). Longitudinal section. (40x)
(b). Transversal section. (40x)

Yamaguchi and Nakajima (1973) found also that endogenous cytokinin concentrations in sweetpotato were necessary for adventitious root formation, but even low concentration was sufficient for adventitious shoot production. Sihachakr *et al.,* 1997 indicated that some portions of sweetpotato compact callus induced onto initiated medium differentiated meristematic areas, whereas, transfer of callus to media containing a high level of zeatin resulted a successful formation of shoots in only two from ten evaluated cultivars. Also, Vishnevetsky *et al*, 2003 observed that medium supplemented with different combinations of NAA and BA has endorsement effect on *de novo* bud formation of *Nerine sarniensis*. In addition, Chen *et al.* (2006) observed that sweetpotato callus produced shoot buds when transferred to regeneration medium without growth regulators.

Conclusion:

In this study, induction of regenerative callus was successful onto hormone free initiation medium treatment (HFIM) with high significant difference than picloram initiation medium treatment (PIM). Enriched cytokinin regeneration medium (BARM) scored the highest significant increase in number of regenerated shoots per shooted callus and frequency of regenerated shoots. The interaction between the initiation medium and regeneration medium was found significant. The best results were obtained with BARM under hormone free initiation medium (HFIM). Callus sections exhibited the formation of *de novo* meristemoids shoots. Shoots rooted well and grew vigorously when subcultured on free hormone rooting medium (RM). Regenerated plantlets were then transferred into pots in controlled growth chamber incubator and successfully established in the greenhouse.

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