Plant Omics Journal
2(5):190-196 (2009)
ISSN: 1836-3644

Effect of $FeSO_4$ and pH on shoot regeneration from the cotyledonary explants of Tossa Jute

[1]Huda, K.M.K., [2]Bhuiyan, M.S.R., [2]Zeba, N., [2]Banu S.A., [2]Mahmud, F. and [3] Khatun A.

[1&2] Department of Genetics and Plant Breeding, Faculty of Agriculture, Sher-e-Bnagla Agricultural University, Dhaka-1207, Bangladesh
[3] Chief Scientific Officer (CSO), Genetic Resources and Seed Division, Bangladesh Jute Research Institute (BJRI), Dhaka-1207, Bangladesh

Corresponding author:mashnuk@yahoo.com

Abstract

Contamination free healthy seedling is required for plant regeneration. In order to achieve this, seeds of tossa jute were germinated on both agar supported hormone free MS medium and cotton supported hormone free liquid MS medium *in vitro*. Percentage of seeds germinated on cotton supported medium was found to be much higher (97.6%) than seeds germinated on agar (45.8%) supported medium. Seedlings grown on cotton supported medium were healthier than the seedling grown on agar supported medium. To optimize the $FeSO_4$ concentration and pH level for *in vitro* shoot regeneration from the cotyledon explants of tossa jute (var. O-9897 and O-72), the explants were inoculated onto MS medium (0.5 mg/l IAA and 3.0 mg/l BAP) which was maintained with different concentrations of $FeSO_4$ (0, 28, 56, 84 and 112 mg/l) and a wide range of pH levels (4.0 to 7.5). The highest rate of shoot regeneration was achieved in a shoot-regenerating medium with 56 mg/l $FeSO_4$ and a pH of 5.5. Shoot multiplication and growth rate were significantly affected by different concentrations of $FeSO_4$. Shoots were not formed in a $FeSO_4$ free medium. Percentage shoot regeneration and number of shoots produced per explant were significantly affected by the pH but both variety respond well in pH 5.5. When the pH was adjusted below or above 5.5, the growth rate of the shoot significantly decreased.

Keywords: *C. olitorius*; cotton-supported MS medium; genotype; micropropagation; multiplication; morphogenesis

Introduction

Tissue culture techniques have become an attractive field of biotechnological research. The benefits of these studies are particularly valuable in the areas of large-scale clonal propagation, crop improvement, the production of important plant compounds and the conservation of genetic resources. The development of shoot regeneration efficiency requires a better understanding of the influence of culture conditions on shoot regeneration. Ferrous sulphate is the main source of iron and sulpher energy for *in vitro* cultures. Plant cells and tissues in a culture medium lack autotrophic ability and therefore, need external $FeSO_4$. The addition of an external iron and sulpher source to the medium enhances the proliferation of cells and regeneration of green shoots. The optimal $FeSO_4$ concentration in a medium should be sufficient to satisfy the basic energy requirements for cell division, differentiation and not impose any negative osmotic effects on shoot formation. This indicates that different concentrations of $FeSO_4$ are one of the factors controlling the induction and growth of shoots. The growth of the shoots also affected by the different concentration of sucrose (Gibson, 2000; Gurel and Gulsen, 1998). Plant cells and tissues require an optimum pH for growth and development in cultures. The pH affects nutrient uptake as well as enzymatic and hormonal activities in plants (Bhatia and Ashwath, 2005). The optimal pH level regulates the cytoplasmic activity that affects cell division and the growth of shoots and it does not interrupt the

Table 1. Combined effect of variety and culture media on seed germination in tossa jute

Variety	Culture media	No. of seed germinated	Seed germination (%)
O-9897	Cotton	46.2b	92.4b
	Agar	21.7c	43.1c
	Cotton	48.8a	97.6a
O-72	Agar	22.9d	45.8d
Level of significance		**	**
LSD0.05		0.916	1.83

function of the cell membrane and the buffered pH of the cytoplasm (Brown et al., 1979). The changes in external pH have a small transient effect on cytoplasmic pH but the cells are readily readjusted towards their original pH (Parton et al., 1997), thus the effect of external pH on cytoplasm is not long lasting. However this change may affect plant growth as follows. Exposure of cells to extreme low pH leads to conversion of inorganic phosphate into organic phosphate at the extracellular region. This is also accompanied by a reduction in ATPs which leads to reduced plant growth (Mimura et al., 2000). The detrimental effects of adverse pH are generally related to an imbalance in nutrient uptake rather than to direct cell damage. The pH also influences the status of the solidifying agent in a medium: a pH higher than 6 produces a very hard medium and a pH lower than 5 does not sufficiently solidify the medium (Bhatia and Ashwath, 2005). Therefore, it is necessary to optimize the $FeSO_4$ concentration and pH level for maximum shoot regeneration because the $FeSO_4$ concentration and pH level directly influence shoot regeneration.

In the present study, cotyledons of tossa jute were cultured in a MS medium with various $FeSO_4$ concentrations and pH levels. Based on the obtained results, optimum $FeSO_4$ concentration and pH level were determined that affected to the efficient shoot regeneration from cotyledons of tossa jute.

Materials and methods

Germination of seeds

Seeds of tossa jute (vars. O-9897 and O-72) were surface sterilized by immersion in 0.1% (w/v) mercuric chloride for 20 min, followed by 4-5 washes with deionized water. All the seeds were placed on the surface of the 50 ml aliquots of hormone free agar solidified (0.8%, w/v) MS basal medium (Murashige and Skoog, 1962) in 100 ml conical flasks. In another set of experiment, surgical cotton (1 gm approx. in each flask) was used instead of agar in association with MS basal liquid medium to obtained optimum seedling production. Each flask contained 20 ml of hormone free MS liquid medium. Cultures were placed in a growth room with 20°C under 1.0 Wm^2 of daylight fluorescent tubes with 12h photoperiod. Seed germination percent and number of healthy seedling was recorded.

Explants

Cotyledons (with attached petioles) of 7-8 days old *in vitro* grown seedlings of *C. olitorius* were excised and cultured on MS agar (0.8%, w/v) solidified medium supplemented with 0.5 mg/l IAA and 3 mg/l BAP. Explants were aseptically placed with the abaxial (lower) surface touching the medium. The culture flasks (250 ml conical flask) were incubated in a controlled environment which was maintained at 28°C under a Wm^2 of day light fluorescent tubes with 12h day length.

Culture media

All the media including the control were supplemented with 0.5 mg/l IAA and 3 mg/l BAP and solidified with 0.8% agar. Cotyledon with attached petiole was used far examining the effects of the $FeSO_4$ concentration on shoot regeneration. Five different concentrations (0, 28, 56, 84 and 112 mg/l) of $FeSO_4$ in a medium were employed for this experiment. On the other hand, same explant was used to examine the effects of pH on shoot regeneration. The pH of the medium was varied from 4.0 to 7.5 (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5) and the pH of the media was adjusted (prior to autoclaving) by using either 1M NaOH or 0.25 M HCl. To minimize condensation, the media were cooled to 40°C before the lids were tightened. No pH buffers were used.

Observations

Observations were recorded after six weeks of inoculation and these included percent seed germination, percent shoot regeneration, number of shoot produced by per cotyledon and days required for shoot regeneration.

Table 2. Shoot regeneration from cotyledonary petioles in MS media containing different concentration of $FeSO_4$

$FeSO_4$ (mg/l)	No. of explants showing shoot	Shoot regeneration (%)	Days required for shoot regeneration
0	0.00d	0.00d	0.00c
28	6.67b	55.55b	7.00a
56	9.83a	81.94a	6.50b
84	6.50b	54.16b	6.50b
112	4.00c	33.33c	7.00a
Level of significance	**	**	**
LSD 0.05	1.30	10.84	0.012

Experimental design and statistical analysis

All the tubes were incubated in a controlled environment room according to a completely randomized design with three replication. Analysis of variance for different component data was performed and Least Significant Difference (LSD) at 0.05 level of probability was used to the means. This experiment was studied in the Genetic Engineering Laboratory, Cytogenetic Department, Bangladesh Jute Research Institute (BJRI) Dhaka during the period of 2005-2006.

Results

1. Germination of seeds

Seeds were germinated from the varieties of *C. olitorius* (O-9797 and O-72) on both clinical cotton and agar supported MS solidified medium. The percent of seeds germination in agar medium found to be very low (43.1%), as compared to clinical cotton supported MS liquid medium, which was found to be very high (97.6%) (Table 1). The seedlings of tossa jute (*C. olitorius*) varieties grown on clinical cotton supported medium were found to much healthier than the seedlings grown on agar supported medium.

2. Effect of different concentration of $FeSO_4$ on shoot regeneration

No shoots was regenerated in the MS medium without $FeSO_4$ (Table 2). On the other hand, the percentage of shoot formation and number of explants producing shoots increase with the increase of $FeSO_4$ concentration in the medium upto 56 mg/l, except for the character days required for shoot regeneration. However, the performance of shoot development gradually decreased with an increase in $FeSO_4$ concentration to above 56 mg/l. The medium with 56 mg/l $FeSO_4$ showed the highest percentage of shoot formation (81.94%) and total number of shoot per culture (9.83 shoots). The combined effect of varieties and different concentration of $FeSO_4$ had also showed statistically significant difference for the characters studied in this experiment (Table 3). The variety O-9897 and O-72 produced highest percentage of shoot formation 80.55% and 83.33% respectively when the medium supplemented with 56 mg/l $FeSO_4$. On the contrary, no shoot was formed when the medium contained 0 mg/l $FeSO_4$. However, the variety O-72 showed best responses as compared to the variety O-9897.

3. Effect of different concentration of pH on shoot regeneration

pH had statistically significant effect on percent shoots regeneration response. However there was a trend such the better shoot regeneration occurs at the acidic pH (4.5-6.0) rather than the alkaline (6.5-7.5) (Table 4). The optimal pH was 5.5 at which 65.30% of the explants produced shoots. Statistically significant (0.48) effect was observed for the number of shoots produced per explants. The highest number of shoots/cotyledon (10.00) was recorded in pH level 6.0 the lowest number of shoot/cotyledon (5.4) was recorded when the medium was adjusted to pH 4.0 and 7.5 respectively.

The effect of varieties and pH level was also showed statistically significant difference in respect of percent shoot regeneration and number of shoots produced per cotyledons (Table 5). Both the variety O-9897 and O-72 produced highest percent shoot regeneration 68.20% and 62.40% respectively when the medium contained pH 5.5, but the maximum number of the shoots produced per cotyledon (12.00) was counted for the variety O-72 at the pH level 6.0, on the other hand, the lowest percent of shoot formation (32.60) and number of shoot produced by each cotyledon (5.00) was recorded for the variety O-9897 with pH level 7.5.

Discussions

Germination percentage of seeds in cotton supported liquid medium was higher than the agar medium and it also produced healthier seedling. Similar finding

Table 3. Combined effect of variety and different concentration of $FeSO_4$ on shoot regeneration

Variety	$FeSO_4$ (mg/l)	No. of explants showing shoot	Shoot regeneration (%)	Days required for shoot regeneration
O-9897	0	0.00	0.00	0.00
	28	6.00	49.99	7.00
	56	9.67	80.55	6.00
	84	7.00	58.33	7.00
	112	4.00	33.33	7.00
O-72	0	0.00	0.00	0.00
	28	7.33	61.11	7.00
	56	10.00	83.33	7.00
	84	6.00	49.99	6.00
	112	4.00	33.33	7.00

was reported for seed germination of white jute varieties by Khatun et al., (2001-2002). This result could be a valuable addition for tissue culture system as cotton supported seed germination system was comparatively cheaper than the agar supported system as agar was very expensive commercially; whereas, the price of cotton is negligible compared to research grade agar. Research grade agar 500 g will cost Taka 4000.00 (approx) whereas, one kg surgical cotton cost only Taka 60.00 in the local market, not only this it also required comparatively lesser (i.e. 20 ml/flask) amount than agar supported media 50 ml/ flask.

Supplementation of culture medium with 56mg/l significantly improved growth and plant regeneration from cotyledon of tossa jute. As the concentration increase shoot regeneration and days required for shoot regeneration was gradually decrease. In contrast, for *Corchorus capsularis* (Jute), supplementation of culture medium with 0.5% [w/v] Pluronic F-68 also enhanced shoot regeneration from cotyledonary explants, coupled with increases in both cotyledon and shoot biomass (Khatun et al.,1993). Interestingly, Pluronic F-68 has also been shown to stimulate *in vitro* growth when added to medium for nodal explants of the tropical woody species, *Manihot esculenta* (Cassava) (Konan et al., 1997). Whilst the precise mechanism of the beneficial effects of $FeSO_4$ and Pluronic F-68 in plant cell and tissue cultures has yet to be elucidated, there is speculation that, at lower concentrations, the surfactant transiently increases plasma membrane permeability and, hence, cell accessibility to nutrients, phytohormones and, perhaps, oxygen (Lowe et al., 1993). Such effects on cell membranes may involve changes in lipid-lipid and/or lipid/protein interactions (Lowe et al., 1993). Medium supplementation with Pluronic F-68 enabled the phytohormone, thidiazuron, to be used at a 10-fold lower concentration than that normally effective at facilitating shoot regeneration in *Populus* (Noel et al., 2002). When used at higher concentrations, $FeSO_4$ may cause detrimental, irreversible changes to cell membranes (Lowe et al., 1993; Noel et al., 2002).

The pH of the culture medium is an important factor far proliferating shoots *in vitro.* In the absence of pH regulation, the ionization of acidic and basic groups causes considerable changes in structure that affect their function at the cellular level (Sakano, 1990). The present study suggested that shoot regeneration is affected by the changes in the media pH. These results suggested that tossa jute is tolerant to a wide range of pH. This response in tissue culture can be compared to that in the field where jute are successfully grown in soils having a wide range of pH (Mills, 2001). Every species requires an optimum pH which can promote maximum shoot formation. In the present study, a batter performance in all parameters on shoot development was found at pH 5.5 in a MS medium containing 0.5 mg/l IAA and 3mg/l BAP.

Jute tissues are able to tolerate a broad range of pH in two ways. Acidic pH is tolerated by exporting out the protons (H+) from the cytoplasm to the extracellular space in exchange for anions, or the cells growing in an acidic environment degrade cytoplasmic organic acid to raise the pH (George, 1993). Conversely, alkaline pH is tolerated by the synthesis of organic acids, such as malate from a neutral precursor (Findenegg et al., 1986). These mechanisms are likely to ensure that tossa jute plants tolerate a range of pH in *in vitro* culture.

The changes in pH during culture can be explained by the differential uptake of nitrogen sources; the uptake of NO^{3-} leads to a drift towards an alkaline pH, while the uptake of NH^{4+} results in a shift towards acidity (George, 1993). The pH drop may

Table 4. Shoot formation for cotyledonary petioles of tossa jute in MS media containing different pH level

pH level	Shoot regeneration (%)	No. of shoots/cotyledon
4.0	35.15e	5.40e
4.5	41.70d	7.50c
5.0	47.80c	9.00b
5.5	65.30a	9.70a
6.0	58.60b	10.00a
6.5	48.95c	7.60c
7.0	42.10d	6.20d
7.5	35.70e	5.40e
Level of significance	**	**
LSD 0.05	2.80	0.48

Table 5. Combined effects of variety and different pH level on percent shoot regeneration and number of shoot/cotyledon

Variety	pH level	Shoot regeneration (%)	No. of shoot/ cotyledon
O-9897	4.0	35.50gh	5.40gh
	4.5	40.80fg	8.00de
	5.0	47.40de	8.80d
	5.5	68.20a	9.20c
	6.0	56.20c	8.00de
	6.5	45.50e	6.60f
	7.0	41.40f	5.40fgh
	7.5	32.60gh	5.00gh
O-72	4.0	34.80gh	5.40gh
	4.5	42.60efg	7.00ef
	5.0	48.20de	9.20c
	5.5	62.40b	10.20b
	6.0	61.00b	12.00a
	6.5	52.40d	8.60d
	7.0	42.80ef	7.00ef
	7.5	38.80g	5.80fg
Level of significance		**	**
LSD 0.05		3.97	0.68

lead to preferential uptake of cations (Singha et al., 1987) and to the exudation of organic acids from plant materials (Singha et al., 1987; Yoshihara and Hanyu 1992). However, Williams et al. (1990) reported that changes occur in the pH of *in vitro* nutrient media during preparation and over the culture period. The direction and extent of the changes depend upon the initial pH and the presence or type of gelling agent. Agar-based medium was progressively acidified in the presence of a living *Ptilotus exaltatus* explants which was not a response to wounding. Martin and Rose (1976) reported that the uptake of ammonium and sugar by *Ipomea* cell suspension was lower at pH 4.8 than at pH 5.6.

At low pH, cells release H^+ to the extracellular environment affecting the absorption of nutrients, especially the $NH4^+$ (Schubert et al., 1990). At high pH, cells release OH^- ions thus the absorption of NO_3^- is adversely affected (Muhlestein et al., 1999).

The proliferation of *Azadirachta indica* shoots significantly increased when the pH of the culture medium was adjusted to 5.8 before autoclaving (Gautam et al., 1993). Meanwhile Nair and Seeni (2003) also found the best shoot multiplication at pH 5.8 level in a medicinal plant, *Calaphyllum apetalum*. Lower and higher pH levels showed low performance for the induction and elongation of shoots from leaf-derived calli of *A. elata.* The main reason for these

results seems to be the solid status of the medium: a higher pH level resulted in a hard medium, while a lower pH resulted in unsatisfactory solidification of the agar. This also agrees the results of a study of *Amygdalus cammunis* (Gurel and Gulsen, 1998). In this study, the pH level played an effective role in enzyme and growth regulator activities that affect the function of cells as well as whole plants. The role that optimum pH plays in enhancing the activities of growth regulators and enzymes have been suggested earlier by Faisal et al. (2006).

In conclusion, the results of this experiment demonstrated that *C. olitorius* showed a very high degree of seed germination on clinical cotton supported media compared to agar solidified medium. This system also ensures a very healthy and diseases free seedling production. This result could be a valuable addition for jute tissue culture system as cotton supported seed germination system is comparatively cheaper compared to agar supported system. Supplementation of culture medium with 56 mg/l $FeSO_4$ showed a marked stimulation of shoot production from tossa jute explants. Both differences in number of shoot production cotyledon-1 and percentage of explants producing shoot has been increased in the presence of $FeSO_4$ (upto 56mg/l) without showing any morphological abnormality. Jute tissues can tolerate a wide range of pH as its shoot response; number of shoots produced per explant was not affected when the media contained pH level upto 5.5-6.0. Shoot regeneration reduced at lower and higher pH indicating that the pH was influencing jute growth indirectly via possibly affecting nutrient uptake at both extremes of pH. Further research is needed to elucidate the mechanism by which $FeSO_4$ or either the low pH or high pH affects growth in tossa jute and to determine whether other jute cultivars respond similar to the tossa cultivar.

Acknowledgements

Authors are highly thankful to Vice Chancellor, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh for appropriate funding and we also pay our sincere thank to the Director General, Bangladesh Jute Research Institute (BJRI), and Dhaka, Bangladesh for providing laboratory facilities and other logistic support.

References

Bhatia P, Ashwath N (2005) Effect of medium pH on shoot regeneration from the cotyledonary explants of Tomato. Biotechnology 4: 7-10

Brown DCW, Leung DWM, Thorpe TA (1979) Osmotic requirement for shoot formation in tobacco callus. Physiol Planta 46: 36-41

Faisal M, Siddique I, Anis M (2006) *In vitro* rapid regeneration of plantlets from nodal explants of *Mucuna pruriens.* A valuable medicinal plant. Ann Appl Biol 148: 1-6

Findenegg GR, Beusichem MLV, Keltjens WG (1986) Proton balance of p1ants: physiological, agronomical and ecological implications. Netherlands J Agril Sci 34: 371-379

Gautam VK, Nanda K, Gupta SC (1993) Development of shoots and roots in anther-derived callus of *Azadirachta indica* A. juss. A medicinal tree. Plant Cell Tiss Org Cult 34: 13-18

George EF (1993) The Components of Culture Media. In: George E (ed) Plant Propagation by Tissue Culture. Exegetics Lid. England, p 274-338

Gibson IS (2000) Plant sugar-response pathways. Part of a complex regulatory web. Plant Physiol 124: 1532-1539

Gurel S, Gulsen Y (1998) The effects of different sucrose, agar and pH levels on *in vitro* shoot production of Almond (*Amygdalus communis* L.). Tr J Bot 22: 363-373

Khatun A, Mahboob S, Alam MS (2001-2002) Attempts to obtain plant regeneration system from *C. olitorius*. Annual Report. Bangla. Jute Res. Inst. Manik Mia Avenue, Dhaka, Bangladesh. P 60-63

Khatun A, Davey MR, Power JB, Lowe KC (1993) Stimulation of shoot regeneration from jute cotyledons cultured with non-ionic surfactants and relationship to physicochemical properties. Plant Cell Rep 13: 49−53

Konan N, Schöpke KC, Carcamo R, Beachy RN, Fouquet TC (1997) An efficient mass propagation system for cassava (*Manihot esculenta* CRANTZ) based on nodal explants and axiliary bud-derived meristems. Plant Cell Rep 16: 444−449

Lowe KC, Davey MR, Power JB, Mulligan BJ (1993) Surfactant supplements in plant tissue culture systems. Agro-Food Ind. Hi. Tech 4: 9−13

Martin S, Rose D (1976) Growth of plant cell *(Ipomea)* suspension cultures at controlled pH levels. Can J Bot p: 54

Mills HA (2001). Tomato. University of Georgia. USA

Mimura T, Shindo C, Kato M, Yokota E, Sakano K, Ashihara H, Shimmen T (2000) Regulation of cytop1asmic pH under extreme acid conditions in suspension cultured cells of *Catharanthus roseus*: a possib1e ro1e of inorganic phosphate. Plant and Cell Physiol 41: 424-431

Muhlestein DA, Hooten TM, Richard K, Grossl P, Bugbee B (1999) Is nitrate necessary to Biological life support? www.usu.edu/cpl/Dawn's%20ICES%

20paper% 201999 .pdf Date of Access 10-6-2003.
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioasssays with tobacco tissue culture. Plant Physiol 15:473-497
Nair LG, Seeni S (2003) *In vitro* multiplication of *Calophyllum apetalum* (Clusiaceae), an endemic medicinal tree of the Western Ghats. Plant Cell Tiss Org Cult 75: 169-174
Noel N, Leple JC, Pilte G (2002) Optimization of *in vitro* micropropagation and regeneration for *Populus* x *Interamericana* and *Populus* x *Euramericana* hybrids (*P. deltoides*, *P. trichocarpa*, and *P. nigra*). Plant Cell Rep 20: 1150−1155
Parton RM, Fischer S, Malho R, Papasouliotis O, Jelitto TC, Leonard T, Read ND (1997) Pronounced cytoplasmic pH gradients are not required for tip growth in plant and fungal cells. J Cell Sci 110: 1187-1198
Sakano K (1990) Proton/phosphate stoichiometry in uptake of inorganic phosphate by cultured cells of *Catharanthus roseus* (L.) G. Don. Plant Physiol 93: 479-483
Schubert S, Schubert E, Mengel K (1990) Effect of low pH of the root medium on proton release, growth and nutrient uptake of fie1d beans *(Vicea faba).* Plant Soil124: 239-244
Singha S, Oberly GH, Townsend EC (1987) Changes in nutrient composition and pH of the culture medium during *in vitro* shoot proliferation of crabapple and pear. Plant Cell Tiss Org Cult 11: 209-220.
Williams RR, Taji AM, Winney KA (1990) The effect of Ptilotus plant tissue on pH of *in vitro* media. Plant Cell Tiss Org Cult 22: 153-158
Yoshilhara T, Hanyu H (1992) pH changes in culture medium with progress of growing stages, callus, multiple shoot and intact plant of strawberry. Acta Hort 319: 291-296