International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X
www.ijpsi.org Volume 4 Issue 8 ǁ August 2015 ǁ PP.07-12

Standardization and establishment of an efficient protocol for *in vitro* multiplication of Strawberry plant and its genetic stability testing

Manikuntala Bhandari and Subhash Kanti Roy
PG Department of Biotechnology, Oriental Institute of Science and Technology, Vidyasagar University, Dewandighi, Katwa Road, Burdwan, West Bengal, India

ABSTRACT : *In vitro multiplication of Strawberry plant and its genetic stability testing were studied to standardize and establish an efficient protocol. Out of three basal media (MS, B_5 & NN), MS showed the best response. Very little and no response was observed in B_5 and NN respectively. MS basal medium supplemented with different combination and concentration of growth regulators (NAA, BAP & GA3) showed variable response in in vitro multiplication of strawberry plant. Equal concentration (5mg/l) of NAA and BAP each and low concentration (0.5mg/l) of GA3 showed maximum response (86%) with maximum number of shoot bud proliferation (60 per explant). Increase in growth regulator concentration decreased the shoot multiplication response. No rooting was observed in auxin free media. MS media supplemented with IBA (0.5mg/l) and IAA (0.5mg/l) initiated better rooting. Genetic stability testing was performed by isozymic analyses (esterase, peroxidase, α-amylase and acid phosphatase) of the in vivo and in vitro grown plant tissue using crude enzyme/ total protein as a starting sample. Among the four isozymes tested, esterase and acid phosphatase showed high polymorphism and two others were low in polymorphic band. The isozyme profiling of both in vivo and in vitro plants exhibited common banding pattern indicating their same genetic fidelity.*

KEYWORDS : *Genetic Stability, In vitro Multiplication, Isozyme Analysis, Strawberry.*

I. INTRODUCTION

The Strawberry, *Fagaria ananassa* Duch. (Rosaceae), is an important and popular fruit crop for its fragrance, taste, nutritional properties and commercial value in food industries. In addition, it exhibits various medicinal properties including high level of antioxidant capacity/ activity[1,2]. Strawberry is produced mainly in temperate and subtropical climates. There are more than 20 *Fragaria* species and numerous cultivars commercially cultivated in almost 37 countries[3]. Since strawberry cultivation is not popular in all countries due to unpredictable climate conditions and lack of proper cultivars, use of micropropagation technique can play a vital role to produce large number of true-to-type plants from a meristematic tissue in a relatively short period of time and space[4,5]**.** Thus *in vitro* micropropagation is considered as important tool for crop improvement in plant breeding since it has many advantages over conventional propagation methods[6]. Commercial production of strawberry using *in vitro* micropropagation process bears several risks. Plant off-types and genetically not identical to the mother plant may be among the resulting plants[3]. *In vitro* production of plants involves the application of plant growth regulators, such as auxins and cytokinins. Changes to these phytohormone habituations known to be associated with genetic instability in plant[7,8]. The failure to observe gross changes or abnormalities in morphology of plants does not negate the possibilities of genetic variation which careful and specific analyses might revel. Unambiguous identification is especially important in clonally-propagated crops such as strawberry[9]**.** Commercial cultivators are needed to be sure that they are investing their right kind and money in propagating the specific cultivar that they have chosen on the basis of yield, harvest time, size and shape[3]. Presently there are various methods available which can be used to detect and monitor tissue culture-derived plants and cultivar identification. One of the techniques, isozyme analysis is a molecular marker system, based on the staining of protein with identical function but, different electrophoretic mobilit[10,11]. Through this analysis, genetic stability of *in vitro* collection is being periodically evaluated[12]. Apart from being inexpensive and results in less exposure to toxic chemicals, isozyme electrophoresis has led to cultivar identification and solve various problems of plant taxonomy in order to distinguish or to confirm species[11].

Strawberry is a highly valued fruit crop and availability of its elite germplasm and cultivation in West Bengal is a tremendous problematic one. Micropropagation is the right candidate for continuous supply of true-to-type germplasm irrespective of the seasonal variation. Since tissue culture derived plantlets possess somaclonal variation, checking the genetic fidelity between *in vivo* and *in vitro* plant is most important for

varietal identification. A very few works on *in vitro* micropropagation of strawberry in West Bengal are on record[13]; but there are scanty reports regarding micropropagation of Strawberry plant followed by its genetic fidelity testing in India as well as in West Bengal. Isozyme analysis is considered to be a simple, efficient and inexpensive technique than PCR based fingerprinting[11] for genetic fidelity testing.

Keeping this background information the present study has been initiated to produce an efficient and reproducible protocol for *in vitro* production of strawberry plant using MS, B_5 and NN basal media. Emphasis was given on MS and B_5 basal media supplemented with different combination and concentration of plant growth regulators followed by its genetic fidelity testing using Isozyme analyses.

II. MATERIAL AND METHODS

Strawberry **(***Fragaria ananassa* Duch) plants were collected from the agricultural farm of Oriental Institute Of Science and Technonogy (OIST), Burdwan, WB. The tender meristematic portions (shoot apices and nodal segments) of the mother plants were taken as explant for *in vitro* studies of strawberry plant.

***In vitro* multiplication**

Suitable explants (shoot apex and nodal segment) were surface sterilized using standard protocol[13] and initially inoculated in MS (Murashige and Skoog, 1962)[14], B_5 (Gamborg's et al., 1968)[15] and NN (Nitsch and Nitsch, 1969)[16] basal media. On the basis of the best response MS and B_5 basal medium with different combinations and concentrations of growth regulators were used subsequently. The cultures were kept in plant tissue culture laboratory with standard cultural condition under 16 hours photoperiod (40-80µ mole m^{-2} s^{-1}) at 25^0C $\pm 1^0$C and 78% relative humidity[17]. The steps followed in *in vitro* micropropagation were preparation of nutrient media, culture initiation, sub culturing, rooting and hardening stage. Rooting was performed by subculturing the plantlet (shoot fully developed) in MS media containing IAA and IBA both or alone at various concentrations. Hardening of fully developed plantlet (shoot and root well established) was carried out after removing them from culture vessel followed by thorough washing the root lets to free from Agar particle, then transplanted into pots containing a soil mixture : sand (2:1). The pots were enclosed in polyethylene bags to minimize moisture loss. The bags were opened gradually for conditioning to greenhouse environment (Fig 1:F & G).

Isozyme analysis

Isozymic profiling of four enzymes-- esterase (EST, E.C.3.1.11), peroxidase (PRX, E.C.1.11.1.7), α-amylase (E.C.3.2.1.1.) and acid phosphatase (ACP, E.C.3.1.3.2) following standard protocol[18] was investigated. Crude enzyme/ protein extracts of tissue culture generated plant (1st, 2nd, and 3rd generation) and *in vivo* plant were done using 2g of meristematic portion following the standard protocol[18]. The extracted crude enzymes/ proteins were estimated by the Folin-ciocalteu method[19]. The protein extracts were then subjected to resolve in Native PAGE electrophoresis. After electrophoresis the gel was prepared for isozymic staining using the standard method[18]. Isozymic profiling was documented.

Statistical analysis

Mean, Standard Error of Mean (SE) & Coefficient of variance (CV) of all *in vitro* multiplication data have been analyzed.

III. RESULTS AND DISCUSSION

***In vitro* multiplication**

In vitro investigation was conducted using shoot apex and nodal segment as explants in three basal media viz., MS, B_5 and NN. Shoot apex with MS basal medium showed best response (Table 1, Fig.1) and relatively very poor and no response was observed in B_5 (Table 2, Fig. not shown) and NN basal medium respectively. MS and B_5 basal media with different concentrations and combinations of Auxin (NAA), Cytokinin (BAP) and Gibberellin (GA_3) were studied extensively . The MS medium with growth regulators (NAA, BAP and GA_3) played a key role in shoot induction as well as multiple shoot initiation. Observations were taken after 28 days of inoculation. Explants' response, initiation of shoot bud, number of shoot bud per explant and shoot length data were noted. This study reflected that the low concentration of Auxin and Cytokinin with no Gibbrrelin rendered poor response. However, different concentrations of NAA, BAP,and GA3 showed variable response in *in vitro* culture of strawberry plant. It was evident that equal concentration of NAA (5mg/l) and BAP (5mg/l) and low concentration of GA3 (0.5mg/l) in MS basal media showed maximum response (86%) with maximum number of shoot bud proliferation (60 per explant) and maximum shoot length (10 cm) as compared to B_5 basal media where poor response (40%) with shoot bud proliferation (38 per

explants) and shoot length (5cm) were observed. Increase in concentration of NAA and BAP decreased the response percentage as well as shoot bud proliferation. The promoting effect of BAP, NAA and GA_3 at suitable concentration in micropropagation of strawberry however had been reported earlier[20,21,22,23]. The best shooting response and highest number of shoot per explant in MS medium containing cytokinin BAP (2mg/l) and auxin NAA (0.1mg/l) had been observer by Mahmoud and Kosar[23]. However, in the present study relatively higher concentration of cytokinin BAP (5mg/l) and auxin NAA (5mg/l) with lower concentration of GA_3 (0.5mg/l) showed the best response. Ara et al.[21] also reported the highest number of shoot per strawberry explant and length of the shoot noted in the medium containing GA_3 (0.5mg/l) and BAP (2mg/l) which was to some extent consistent with the present findings. Previous findings[24] revealed that high concentration of cytokinin reduced the number of micropropagated shoot which was in conformity to the present findings. Similar results had also been reported earlier on papaya[25] as well as *Eucalyptus grandis*[26].

Rooting

It was observed that combined treatment of IAA and IBA in MS media is very much effective for root formation. MS media supplemented with IBA (0.5mg/l) and IAA (0.5mg/l) showed the best result of root formation, length of the root being 3.7cm in this treatment. No rooting was observed in auxin free media. Root induction was optimum in plantlet treated with IBA (1mg/l) and IAA(1mg/l), the average length of the root being 3.1cm and 3.2cm respectively (Table 3, Fig.1). It had been previously reported that combined treatment of IBA (0.1mg/l) and IAA (0.1mg/l) at low concentration showed better root initiation, length of the root being 4.1 cm[27]. Ashrafuzzaman et al.,[28] also reported that concentration of IBA (0.5 mg/l) alone showed good result for root formation, the length of the root being 3.05 cm which corroborates the present findings.

Isozyme analyses

Assurance of clonal fidelity is another important aspect of any successful micropropagation protocol prior to commercialization. PCR-based molecular markers have largely been employed in recent times for various purposes[29,30]. Cost effectiveness is a major checkpoint in any commercial micropropagation programme and assurance of clonal fidelity through any PCR-based detection system definitely appears to be self-contradictory. Efforts, hence, were made to use relatively cheaper profiling technique. Isozymes, though belonging to the second generation of molecular markers[11], were considered to be the right candidate for this purpose as it is a cheaper proposition than PCR-based fingerprinting.

For isozyme analyses, three randomly selected *in vitro* generated plantlets (3rd generation) with that of two *in vivo* plants were analysed. Four widely used isozymes, viz. esterase; peroxidase, α-amylase and acid phosphatase were studied in similar experimental design. Of these four isozymes analysed, esterase and acid phosphatase (fig 2. A & D) showed high polymorphism while the other two (Fig 2:B and C) were low in polymorphic band. The isozyme profiling of both *in vivo* and *in vitro* derived plant showed similar result.

It has been evident from the present study that isozyme profiling of esterase and acid phosphatase showed highest polymorphism as compared to that of peroxidase and α-amylase. Almost similar results were obtained in *Gladiolus* sp. by Roy et al.,[31]. Esterase profiling had also been used to study the genetic stability of micropropagated banana clones[32] in field condition with success.

IV. FIGURES AND TABLES

Figure 1. Stages involved in micropropagation of strawberry using shoot apex as explant (*in vitro* growth):

(A) Initiation, (B) Establishment, (C) Multiplication, (D) Fully developed plant,(E) Rooting, (F) primary hardening and (G) secondary hardening of strawberry plant.

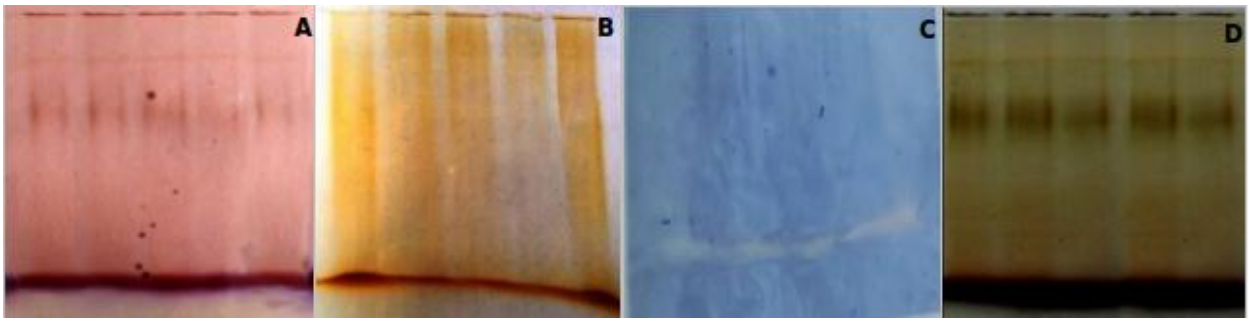

Figure 2. Isozymic profiles (esterase-A; peroxidase-B; α-amylase-C and acid phosphatase-D) of Strawberry plant. Lane 1 and 5 in each gel represents mother plant, while lane 2, 3 and 4 in each gel represents *in vitro* derived strawberry plant (1st, 2nd 1nd 3rd generation respectively)

.

Table 1. Establishment of *in vitro* response of *Fragaria ananassa* (strawberry) using shoot apex as explant : Basal medium MS

Growth regulators (mg/l)			Response (%)	Explants showing initiation of shoot bud (%)	No. of shoot bud/explants	Highest plantlet length (cm)
NAA	BAP	GA_3				
0	0	0	0	0	0	0
0.1	0.5	0	5	3	2	0.1
0.5	1	0	7	8	7	0.2
1	1.5	0.1	12	10	13	0.7
2	2	0.1	15	12	20	1
2	2.5	0.2	19	16	27	3
2	3	0.2	32	28	36	6
2.5	3.5	0.3	45	39	49	7
2.5	4	0.4	58	50	48	7
3.5	5	0.4	56	51	52	8
4.5	5	0.5	82	59	57	9
5	**5**	**0.5**	**86**	**62**	**60**	**10**
5	5.5	0.5	78	56	57	8
5.5	5.5	0.6	79	53	51	7
5.5	6	0.6	72	49	43	6
6	6	0.7	68	43	40	5
6	6.5	0.7	65	38	36	4
6.5	6.5	0.8	63	35	31	4
Mean			46.77	34.00	34.94	4.78
SE (Mean)			6.90	4.77	4.48	0.76
CV (%)			62.56	59.47	54.32	66.95

Table 2. Establishment of *in vitro* response of *Fragaria ananassa* (strawberry) using shoot apex as explant : Basal medium B_5

Growth regulators (mg/l)			Response (%)	Explants showing initiation of shoot bud (%)	No. of shoot bud/explants	Highest plantlet length (cm)
NAA	BAP	GA_3				
0	0	0	0	0	0	0
0.1	0.5	0	3	2	1	0.1
0.5	1	0	4	2	1	0.1
1	1.5	0.1	6	3	1	0.1
2	2	0.1	7	5	2	0.2

2	2.5	0.2	11	8	9	0.3
2	3	0.2	12	8	10	0.5
2.5	3.5	0.3	19	11	14	0.9
2.5	4	0.4	24	20	21	2
3.5	5	0.4	27	25	21	2.5
4.5	5	0.5	32	28	27	3.8
5	**5**	**0.5**	**40**	**36**	**38**	**5**
5	5.5	0.5	37	32	33	4
5.5	5.5	0.6	35	30	31	4.2
5.5	6	0.6	33	27	28	4
6	6	0.7	30	25	24	3.6
6	6.5	0.7	34	30	31	3.6
6.5	6.5	0.8	31	24	21	3.2
Mean			21.39	17.56	17.39	2.12
SE (Mean)			3.11	2.82	2.95	0.42
CV (%)			61.66	68.00	71.82	83.02

Table 3. Root proliferation using different concentrations and combinations of IBA and IAA in MS media

Growth regulators (mg/l)		Average no of roots/explants	Average length of roots (cm)
IBA	IAA		
0.0	0.0	-	-
0.1	-	2	0.7
0.2	-	3	1.0
-	0.1	3	1.2
0.1	0.1	5	2.3
-	0.5	5	1.9
0.5	-	7	3.0
0.5	**0.5**	**10**	**3.7**
-	1.0	8	3.2
1.0	-	7	3.1
1.0	1.0	7	2.9
1.5	1.5	6	2.8
-	1.5	6	2.2
1.5	-	5	2.0
Mean		5.29	2.14
SE (Mean)		0.67	0.28
CV (%)		47.64	48.60

V. CONCLUSION

In vitro investigation of strawberry (*Fragaria annanasa* Duch.) was carried out thoroughly using three plant growth regulators. An efficient micropropagation protocol using shoot apex as explant was developed using NAA (5mg/l), BAP (5mg/l) and GA_3 (0.5mg/l). Hardening of tissue culture generated strawberry plantlet was established using peat moss : sand (2:1 ratio). Genetic fidelity of tissue culture generated plantlet with that of mother plant was tested using, esterase, peroxidase, α-amylase and acid phosphatase profiling. Among the four, esterase and acid phosphatase profiling gave promising response with maximum number of polymorphic band. Upgradation of existing methodology is a continuing practice for any commercially important plant. In plant biotechnology, specially the *in vitro* micropropagation of economically important fruit crops are gaining momentum day by day and it is hoped that the present study will be used for basic and applied research of both academic and industrial purpose of this highly valued fruit crop.

ACKNOWLEDGEMENT

Authors are very much thankful to the Secretary, Oriental Institute of Science and Technology (OIST), Burdwan, WB, for providing research facilities. Thanks are given to Mr. Bikash Khumbhakar for his assistance.

REFERENCES

[1] Wang, H., Cao, G. and Prior, R.L., Total antioxidant activity of fruits. *Journal of Agricultural and Food chemistry,* 1996, **44,** 701-705.

[2] Wang, S.Y. and Jiao, H., Scavenging capacity of berry crops on super oxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen *Journal of Agric. and Food chemistry,* 2000, **48**, 5677-5684.

[3] Gaafar, R.M. and Saker, M.M., Monitoring of cultivars identity and genetic stability in strawberry varieties grown in Egypt. *World J. Agric. Sci.,* 2006, **2(1),** 29-36.

[4] Boxus P., Commercial production of Strawberry plants produced by meristem culture and micropropagation. *Colloques Scientifiques Hort. Abstr*., 1983, **53**: 283-322.

[5] Nehra, N.S., Kartha, K.K. and Giles, K.L, Effect of *in vitro* propagation methods on field performance of two strawberry cultivars. *Euphytiea, 1994,* ***76****: 107- 115.*
[6] Taji, A., Kumar, P.P. and Lakshmanan, P., *In Vitro* Plant Breeding. Food Pro-ducts Press, New York, 2002, pp. 167.
[7] Karp, A., Can genetic instability be controlled in plant tissue culture. *Newslett. Intl. Assoc. Plant Tissue Culture*, 1989, **58**: 2-11.
[8] Phillips, R.L., Kaeppler S.M. and Olhoft, P., Genetic instability of plant tissue cultures;breakdown of normal controls. *Proc. Nat. Acad. Sci., USA*, 1994, **91**:5222-5226.
[9] Kester D.E., The clone in horticulture. *Hort. Sci*, 1983,**75**: 831-837.
[10] Hailu, H.W., Kristiyanto, D.H., Alatawi, A.R.A. and Raquib, S.M., Isozyme Electrophoresis and morphometric comparison of Reed (*Imperata cylindrical*) adaptation to different altitudes. *International Journal of Innovative Research in Science, Engineering and Technology*, 2014, **3(5):**12387-12394.
[11] Zeilder M., Electrophoretic analysis of plant isozymes. *Acta Univ. Palacki. Olouc. Fac. Rerum Nat.Biologica*, 2000, **38**: 7-16.
[12] Smila, K.H., Muthezhilan, R., Radhika, S. and Rajasekarapandian, M., Studies on genetic conformity of two micropropagated medicinal plants using isozymes. *Biotechnol.Bioinf. Bioeng*, 2011, **1(3):** 325-330.
[13] Bhandari, M. and Roy, S.K., Effect of Vermicompost on the growth of strawberry(*Fragaria ananassa* Duch) plant and standardization of micropropagation of elite Germplasm. *International Journal of Engineering Science and Innovative Technology, 2014, **3(6)** : 442-449.*
[14] Murashige, T. and Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 1962 **15**: 473-497.
[15] Gamborg, O.L., Miller, R.A. and Ojima, K., Nutrient requirements of Suspension cultures of Soybean root cells. *Exp Cell res.*, 1968, **50** :151-158.
[16] Nitsch, J. and Nitsch, C., Haploid plants from pollen grains. *Sci.*, 1969, **163** : 85-87.
[17] Gangopadhyay, G., Ro,y S.K., Gangopadhyay, S.B. and Mukherjee, K.K., Agrobacterium mediated genetic transformation of Pineapple Var. Queen using a novel encapsulation-based antibiotic selection technique. *Plant cell Tiss Organ cult*, 2009, **97** : 295-302.
[18] Das, S. and Mukherjee, K.K., Morphological and biochemical investigations on *Ipomoea* seedlings and their species interrelationships. *Ann. Bot*., 1997, **79**: 565-571.
[19] Lowry, O.H., Rose rough, N.J., Farr A. and Randall R.J., Protein measurements with Follin phenol reagent. *J. Biol. Chem.*, 1951, **193**: 265-275.
[20] Popescu, A.N., Issac, V.S., Coman, M.S. and Radulescu, M.S., Somaclonal variation in plants regenerated by organogenesis from callus culture of strawberry (*Fragaria ananassa*). *Acta.Horticulture*, 1997, **439**: 89-96.
[21] Ara, T., Karin, R., Karim, M.R., Ahmad, S., Islam, R. and Hossain, M., Effects of different hormones on *in vitro* regeneration of strawberry *(F ananassa* Duch.). *International Journal of Biological science*, 2012, **2(1) :**93-100.
[22] Bhatt, P.R., Kheroda, Devi, M., Jayalakshmi, H.,Sophia, I. and Prajna, P.S., Effect of plant growth regularors on the establishment and growth of strawberry *(F ananassa* Duch.) var. chandler. *in vitro, Agri. Sci. Research Journal,* 2012, **2(12)** :623-632.
[23] Mahmoud, O. and Kosar, M., Regeneration and Histological of Plants Derived from Leaf explants in vitro Culture of Strawberry, *International Journal of Agricultur and Crop Sciences, 2013, **5-9**: 943-950.*
[24] Hu, C.Y. and Wang, P.J., Meristem shoot tip and bud culture. In: Evans DA, Sharp WR, Ammirato PV and Yamada Y (Eds.), *Hand book of Plant Tissue Culture.Volume. **I**. Macmillan, New York.*, 1983, 177-227.
[25] Cononer, R.A. and Litz, R.E., *In vitro* propagation of papaya. *Hort. Sci.*,1978, **13**: 241-242.
[26] Teixetra, S.L. and De Silva, L.L., *In vitro* propagation of adult *Eucalyptus grandis* Hill Ex. maiden from epicormic shoots (Abstracts VII th.). *Intl. Cong. On Plant, Tissue and Cell Culture (IAPTC), Amsterdam,1990, pp: 218.*
[27] Hasan, M.N., Nigar, S., Rabb,i M.A.K., Mizan, S.B. and Rahman, M.S., Micropropagation of Strawberry (Fragaria ananassa Duch.). *Int. J. Sustain. Crop Prod.*, 2010, **5 (4):** 36-41.
[28] Ashrafuzzaman, M., Faisal, S.M., Yadav, D., Khanan D. and Raihan, F., Micropropagation ofstrawberry *(Fragaria ananassa*) through runner culture, Bangladesh. *J. Agril. Res.*, 2013, **38(3):** 467- 472.
[29] Hao, G., Lee, D.H. and Lee, N.S., A study of taxonomical relationships among species of Korean *Allium* sect. *Sacculiferum* (Alliaceae) and related species using inter-simple sequence repeat (ISSR) markers, *Bot. Bull. Acad. Sin.*, 2002, **43**: 63-68.
[30] Fu, C., Qiu, Y. and Kong, H., RAPD analysis for genetic diversity in *Changium smyrnioides* (Apiaceae), an endangered plant. *Bot. Bull. Acad.Sin.*, 2003, **44**: 13-18.
[31] Roy, S.K. and Gangopadhya, G., Bandopadhyay, T., Modak, B.K., Datta, S. and Mukherjee, K.K., Enhancement of *in vitro* micro corm production in Gladiolus using alternative matrix. *African J. of Biotech.*, 2006, **5(12):** 1204-1209.
[32] Dutta, R., Gangopadhya, G., Das, S., Dutta, B.K. and Mukherjee, K.K., Esterase as a marker to study the genetic fidelity of micropropagated banana, *Biol.Plant.*,2003/ 4, **47**: 421-424.