International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 10, Issue 12, 2018, pp.-1425-1428.
Available online at https://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

Research Article

OPTIMIZATION OF THE TRANSIENT EXPRESSION OF CrSTR IN *Ophiorrhiza mungos* L. PLANTS BY AGRO INFILTRATION METHOD TO ENHANCE CAMPTOTHECIN PRODUCTION

NAGESHA B.V.*[1,2], UGRAIAH A.[1,2], RAVIKANTH G.[3], NATARAJA K.N.[1] AND UMASHAANKER R.[1,2]

[1]*Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bengaluru, 560065, Karnataka, India*
[2]*School of Ecology and Conservation, University of Agricultural Sciences, GKVK, Bengaluru, 560065, Karnataka, India*
[3]*Ashoka Trust for Research in Ecology and the Environment, Royal Enclave, Srirampura, Jakkur PO, Bengaluru, 560064, Karnataka, India, India*
**Corresponding Author: Email - nageshgowdabgh06@gmail.com*

Received: November 18, 2018; Revised: December 26, 2018; Accepted: December 27, 2018; Published: December 30, 2018

Abstract- Camptothecin derivatives are clinically used as anti-tumor indole alkaloids that are currently obtained by extraction from intact plants. In the present study, we developed a method for transient expression of *Catharanthus roseus* strictosidine synthase (CrSTR) gene by Agro infiltration method in *Ophiorrhiza mungos* plant with *Agrobacterium tumefaciens*. Our results indicated that the construct in the LBA 4404 strain imparted a higher transient expression as evident by a higher accumulation of the end product, CPT (0.022 %) compared to the untransformed as well as the mock treatments. These results point at the possibility that overexpression of Strictosidine synthase could in principle lead to the overproduction of strictosidine which then can lead to the higher production of terpenoid indole alkaloids, such as camptothecin.

Keywords- *Camptothecin, Indole alkaloids, Agrobacterium tumefaciens, CrSTR gene*

Citation: Nagesha B.V., *et al*., (2018) Optimization of the Transient Expression of CrSTR in *Ophiorrhiza mungos* L. Plants by Agro Infiltration Method to Enhance Camptothecin Production. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 10, Issue 12, pp.-1425-1428.

Introduction

Plant secondary metabolites have been used in pharmaceutical industry since ancient times as powerful drugs against various chronic diseases. Camptothecin (CPT) is a high-value quinolone alkaloid with the anticancer property. The potentiality of CPT to inhibit the nucleic acid synthesis in mammalian cells makes it an active medicament against ovarian and colorectal cancer. The compound was isolated for the first time by Wall *et al*. [1] while working with the extract of *Camptotheca acuminata* a Chinese tree. Later on, the presence of CPT has been identified in many other plant species irrespective of the family and order. *Nothapodytes foetida* [2], *Chonemorpha grandiflora* [3], *Ervatamia hyneaya* [4], *Merrilliodendron megacarpum* [5], *Pyrenacantha klaineana* [6], and many species of *Ophiorrhiza* [7, 8] are other major sources of CPT. Its reported that, in tree species, the CPT concentration increases as its ages and after a particular point it becomes proportionate to the thickness of bark [9]. The whole plant CPT content of Camptotheca ranges from 0.2 to 5.0 mg/g DW depending on the tissue analyzed [10]. While that of Nothapodytes is reported to be 0.3 mg/g DW [11]. The tree needs 8–10 years to reach the mature stage from seedling and start flowering. It needs 1000–1500 tons of wood chip every year to meet the market demand of CPT [12]. Taking into consideration, that growing and harvesting of herbaceous plants are easier and reliable than tree species, genus *Ophiorrhiza* became more dependable for CPT production. Comparative study on the CPT content in different species of *Ophiorrhiza* genus recorded the highest amount of CPT in species munogs in a range of 0.17–0.5 mg CPT/g DW [13], followed by *O. mungos var*. angustifolia with 0.17–0.47 mg CPT/g DW. Due to the presence of high amount of CPT in the roots, the bulk uprooting of the genus was observed in the recent past which along with the low rate of seed viability and an insufficient number of propagules for vegetative propagation approaches resulted in the destruction of the natural population. In addition to this, the seed progenies are not true to type breed due to its heterozygous nature. These altogether necessitated a sustainable alternative for the production of CPT as well as conserving the plant.

Plant cell tissue and organ culture have been used as an efficient method for commercial production of high-value plant secondary metabolites when natural resources are limited [14,15]. Many studies on in vitro regeneration of different species of *Ophiorrhiza* have been reported by the earlier workers. Cell and tissue cultures of several CPT-producing plants have been investigated as alternative sources. [16] reported the first establishment of cell suspension cultures of *C. acuminata*, although the CPT productivity was practically insufficient [2.5 µgg^{-1} dry weight (DW)]. Callus cultures of *C. acuminate* established by [17] produced comparatively adequate amounts of CPT (2 µgg-1 DW). Calli of *N. foetida* were found to accumulate small amounts of CPT [18, 19]. The first feasible in vitro CPT-production system was developed by transformed hairy roots of *Ophiorrhiza pumila* (Rubiaceae) with *Agrobacterium rhizogenes* [20]. This hairy root culture produced a high level of CPT (1µgg^{-1} DW) and excreted CPT into the culture medium in a relatively large quantity sustained in a scaled-up 3 L bioreactor [21]. Similarly, hairy roots of *C. acuminata*, producing an equal level of CPT, were recently reported [22]. For better production of CPT and further potential genetic engineering, a regeneration protocol of transformed plants of those CPT-producing species is needed. Here, we have developed a method for transient expression of *Catharanthus roseus* strictosidine synthase (CrSTR) gene by Agro infiltration method in *Ophiorrhiza mungos* plant with *Agrobacterium tumefaciens*.

Materials and methods

To standardize the transient expression of CrSTR (Strictosidine synthase), 3 months old *O. mungos* plants were used. The candidate gene strictosidine synthase (STR) has been cloned in many alkaloid producing plants to enhance the production of indole alkaloids. For this study, STR gene constructs prepared from *C. roseus* were obtained from J Memelink [23]. pMOG22-GUSint-TDC-STR [23] and control pMOG22-GUSint [24] procured from Prof. J. Memelink was used as the construct.

Agrobacterium strain and plasmid vector

Agrobacterium strains (LBA 4404 and EHA 105) harboring the vector pMOG22-STR and TDC GusInt was used to induce transient expression in *O. mungos*. The vector contained the hygromycin phosphotransferase (hpt) gene which allows for the hygromycin selection. strictosidine synthase (STR) gene was cloned from *C. roseus*. *Agrobacterium tumifaciens* strains LBA 4404 and EHA 105 was transformed by electroporation and cultured under appropriate growth conditions.

Preparation of competent cells of _Agrobacterium tumefaciens_

A single colony of *A. tumefaciens* (LBA 4404 and EHA 105) was inoculated in 3 ml of YEM medium, incubated at 28°C for 16 hours at 200 rpm and the culture was harvested by centrifugation at 5000 rpm at 4°C. The culture was washed in ice cold water for five to seven times and resuspended in glycerol (60%) and shock frozen in liquid nitrogen and stored at -80°C as competent cells.

Plasmid DNA isolation from _Escherichia coli_

The plasmid DNA was isolated by Sigma Aldrich GenElute Miniprep kit method. The bacterial colony (*Escherichia coli*) harboring the vector pMOG22-STR and TDC GusInt inoculated into 3 ml LB medium with appropriate antibiotics and allowed to grow for 16 hours at 37°C at 200 rpm. Isolated plasmid was confirmed by PCR.

Transformation of plasmid into _Agrobacterium_ through electroporation

A. tumefaciens competent cells were thawed on ice and a 40µl aliquot was transferred to a precooled 2 mm electroporation cuvette. About 1-2µl of plasmid DNA was added to the competent cells and mixed in a electro competent cuvette and electric shock of 1440 volts was applied for 5 second using a gene electroporator (Eppendorf, Germany). After that 800-900µl of YEM media was added to the transferred cells and incubated in an eppendorf tube in a shaker at 200 rpm at 28°C for 4 hours and pelleted down and 850 µl was removed from total 1 ml of YEM media. About 150 µl of *Agrobacterium* cells were plated on YEM medium containing kanamycin (100 mg/l) and rifampicin (10 mg/l) and incubated at 28°C and allowed to grow for 48 hours. Presence of transformed gene in *Agrobacterium* was confirmed by PCR.

Culturing of _Agrobacterium_ strains LBA 4404 and EHA 105 harbouring PMOG22 Gusint-STR and TDC.

The *Agrobacterium* strains LBA 4404 and EHA 105 harbouring pMOG22 Gusint-STR and TDC were cultured on YEM media. About 100 µl of *Agrobacterium* culture were taken from glycerol stock and transferred into 1 ml of YEM media taken in a culture tube containing kanamycin and rifampicin and incubated in the incubator at 28°C for 24 hours. After 24 hours, 1 ml culture was transferred to 2 ml of YEM media containing kanamycin (100 mg/l) and rifampicin(10 mg/l) and incubated in an incubator at 28°C for 24 hours; later these was transferred to 50 ml of YEM media incubated at 28°C for 16 hours.

Agrobacterium infiltration media

The *Agrobacterium* harbouring candidate genes grown in YEM media was pelleted by spinning the culture in a centrifuge at 10000 rpm at 4°C for 10 minutes and resuspended in 20 ml of infiltration media (10 mM MgCl2, 10mM MES, 2% sucrose, 1% glucose and 100 µM acetosyringone). The culture was diluted to OD600 =1 and incubated for 3-4 hours in room temperature. This media was used for transient assay by ago-infiltration method.

Transient expression of CrSTR gene in _Ophiorrhiza mungos_

Three-month-old *O. mungos* seedlings were used for transient assay. The infiltration media was taken in a sterile 5 ml needless syringe, injected into the under surface of the leaf of the plants. A control and mock were also maintained. The transient expression of CrSTR genes was confirmed by GUS assay and HPLC analysis.

GUS Assay

GUS Assay was done 5th and 6th days after agro infiltration. 100 mL of X-Gluc solution was prepared by dissolving 0.052 g of X-Gluc (5-bromo-4-choloro-3-indolyl) in 50 ml of sterile distilled water, Add 5 ml of 1 M sodium phosphate stock solution and 0.1 ml of the detergent Trition X-100. Mixed well and the volume was made up to 100 ml with distilled water. The pH 7 was maintained and the solution was stored in the refrigerator. Agro in filtered leaves were harvested and infected leaf parts were taken and incubated overnight in 2ml Eppendorf containing X-Gluc solution. X-Gluc solution was removed and 70 % ethanol was added to the eppendorf and allowed for 3-4 hours [25].

Estimation of CPT from transient assay

Leaves in which transient expression was performed were harvested and dried in hot air oven at 55°C for 3 days. The dried tissue was ground to fine powder and weighed. 2 ml of 61 % ethanol was added to 100 mg of tissue powder, then sonicated for 15 min and kept on a water bath (60°C) for six hours [26].The samples were kept overnight in a freezer at 4°C and samples were centrifuged at 10000 rpm at 4°C for 10 minutes. The supernatant was collected; filtered and 400 µl of samples was used for HPLC analysis of CPT.

HPLC analysis

Camptothecin was analyzed by reverse phase HPLC (Supelco 516, LC-10AS, and Shimadzu, Japan) on a C18 column (250x4.6mm, 5µm). The HPLC conditions were: 254 nm for CPT as the detector wavelength, 1.5mL/min flow rate and 20µL sample loop. The mobile phase was adjusted as follows: 25% acetonitrile and 70% water + 0.1% Trifluro-acetic acid (TFA) in an isocratic mode [27]. A CPT (Sigma, 95% HPLC purified) standard sample was procured from Sigma Chemicals. The standard was prepared using DMSO and methanol in 1:3 (v/v) ratios respectively. The total analysis run time was min 30; retention time of CPT is at 11.2 min. The presence of CPT in the samples was detected by comparing with the retention time of the standard sample. The area of the standard was compared with area of the sample and the amount of CPT in the extracts was calculated and expressed as percent dry weight of tissue.

Statistical Analyses

All experiments were replicated at least thrice and performed complying to a completely randomized design (CRD). One way analysis of variance (ANOVA) was performed using Microsoft Excel to evaluate the effect of the transient expression of CrSTR on CPT production. Significant difference between means were assessed by Duncan's Multiple Range Test (DMRT) (P = 0.05) [28].

Results

The transformation of *Agrobacterium* was done by electric shock method. The plasmid constructs having pMOG22-GUSint-TDC-STR gene was mobilized into the *Agrobacterium* strains EHA105 and LBA 4404. The presence of strictosidine synthase gene was confirmed by colony PCR in *Escherichia coli* [Fig-1] and *Agrobacterium* strains LBA 4404 and EHA 105 ([Fig-2] were selected for further transient expression studies. To standardize the transient expression protocol in *O. mungos*, 3 months old acclimatized plants grown in green house conditions were selected. The plasmid constructs having pMOG22-GUSint-TDC-STR gene were procured from Prof J. Memelink harboring the CrSTR gene from *Catharanthus roseus*. Transient expression was done by agro infiltration method [Fig-3]. *Agrobacterium* carrying a binary plasmid having pMOG22-GUSint-TDC-STR was infiltrated into leaves of *O. mungos*. Suspensions of particular strains of *A. tumefaciens* carrying plasmid having pMOG22- GUSint-TDC-STR were infiltrated separately. Agro in filtered leaves were harvested on fifth and six day. The GUS assay was done to examine the integration of introduced gene in the agro in filtered leaves. The GUS assay results shows that *Agrobacterium* LBA4404 harboring pMOG22-GUSint-TDC-STR and EHA105 harboring pMOG22-GUSint-TDC-STR induces expression of STR gene in the agro in filtered leaves of the plants [Fig-3]. The leaves were collected after fifth and sixth days after agro infiltration treatment. The leaf extracts were subjected to HPLC analysis for camptothecin estimation. CPT was detected in all treatments (control, mock and infiltrated plants). Agro infiltrated leaves with *Agrobacterium* LBA4404 and EHA105 harboring pMOG22-GUSint-TDC-STR strains showed increase in

Table-1 *CPT estimation from Agro-infiltrated leaves of O. mungos leaves*

Treatments	CPT % (5th day) (Mean ± Sd)	CPT % (6th day) (Mean ± Sd)
Control	0.007^{a} ± 0.0019	0.007^{a} ± 0.0019
Mock	0.012^{b} ± 0.0037	0.009^{a} ± 0.0043
EHA105 STR	0.019^{b} ± 0.036	0.014^{a} ± 0.0016
LBA4404 STR	0.020^{ac} ± 0.036	0.022^{b} ± 0.0043

One way ANOVA; CD = 0.009; means followed by dissimilar superscript are significant at p < 0.01.

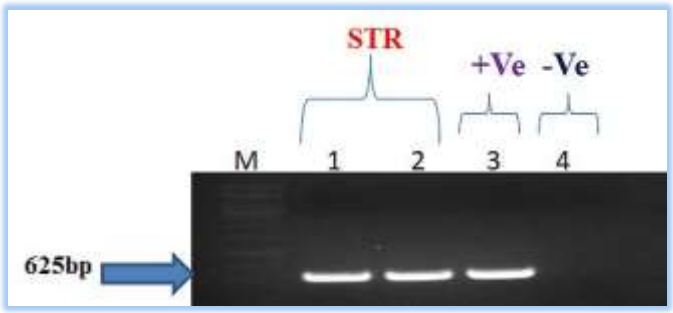

Fig-1 Ethidium bromide stained agarose gel showing STR gene amplification from the colonies of *E. coli*

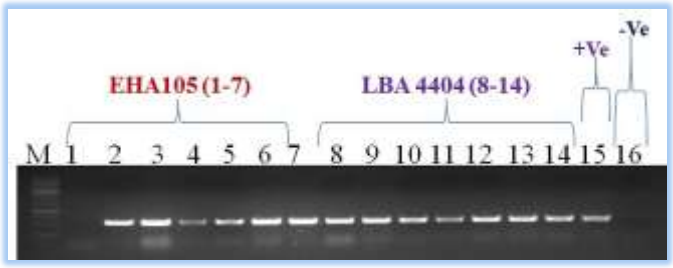

Fig-2 Confirmation of STR gene from colonies of *Agrobacterium* strains EHA105 and LBA 4404 by PCR.

Fig-3 Stages of Transient expression of STR by Agro infiltration method

Fig-4 GUS assay-GUS expression in agro in filtered leaves

camptothecin compared to mock and control [Table-1]. CPT content treated with LBA4404 harboring pMOG22-GUSint-TDC-STR strain produced higher CPT on 5th (0.020 %) and 6th (0.022 %) day compared to EHA 105 5th (0.019 %) and 6th (0.014 %) harboring pMOG22-GUSint-TDC-STR strain [Table-1].

Discussion

Transient expression of genes using *Agrobacterium* is a powerful tool for the analysis of gene function in plants. A transient assay offers advantages over stable transformation including relatively quick analysis of introduced genes and is not generally influenced by the plant genetic constitution [29]. In fact, wherever the development of a stable transformation system in constrained, transient expression system could easily be used to provide the proof of principle of any gene of interest. Using one of the candidate genes of CPT biosynthetic pathway we standardized the protocol for transient expression in *O. mungos*. Construct harboring strictosidine synthase gene from *Catharanthus roseus* (CrSTR) was used in our study. CrSTR was mobilized into two different strains of *Agrobacterium* (EHA105 and LBA 4404). Our results indicated that the construct in the LBA 4404 strain imparted a higher transient expression as evident by a higher accumulation of the end product, CPT (0.022 %) compared to the untransformed as well as the mock treatments [Table-1]. These results point at the possibility that overexpression of strictosidine synthase could in principle lead to the overproduction of strictosidine which then can lead to the higher production of terpenoid indole alkaloids, such as camptothecin. Metabolic engineering involving overexpression of selected genes for the overproduction of plant secondary metabolites has been gaining prominence in recent years [30]. Studies have shown that overexpression of transcription factors and a few regulator genes can improve the production of target secondary metabolites [31]. Earlier reports in *C. roseus*, showed that over expression of strictosidine synthase (STR) and tryptophan carboxylase (TDC) transgenic lines resulted in a ten-fold higher STR activity than wild-type cultures and transformed lines accumulated higher levels of ajmalicine, catharanthine, serpentine, and tabersonine [23]. Hairy root cultures of *C. roseus* over expressing G10H gene and ORCA3 transcription factor using *Agrobacterium rhizogenes* strain MSU440 accumulated 6.5-fold higher catharanthine compared to the untransformed clones [32], reported In Rauwolfia serpentine, overexpression of a Catharanthus tryptophan decarboxylase (CrTDC) gene increased reserpine by 3 folds and ajmalicine by 5 folds [33]. Recent studies in *O. pumila* co-overexpressing genaniol 10 hydroxylase (G10H) and STR caused a 56% increase in the CPT yields compared to non-transgenic lines and single gene transgenic lines, indicating the synergetic effect of the two genes in CPT accumulation. Hairy root lines co-overexpressing G10H and STR produced higher levels of CPT compared to hairy roots and wild-type roots [34]. Admittedly such novel approaches for the production of plant secondary metabolites can greatly relieve the tremendous pressure on natural resources for the extraction of secondary metabolites.

Conclusion

The present study has demonstrated that the study has provided a protocol for the transient expression system for strictosidine synthase (CrSTR) in *O. mungos*. The techniques and results could be of help in developing transgenics of *O. mungos* and overproduction of camptothecin. Transient expression protocol in *O. mungos* has been successfully established by using strictosidine synthase (CrSTR) a key gene involved in CPT pathway. Transiently expressed leaves showed increased CPT production than the wild types. Though preliminary these studies pave way for studying the expression of CrSTR in stable transformation system and its consequence on CPT synthesis. Furthermore, the study indicates the potential of over expression of rate limiting genes such as STR to develop high yielding transgenic lines of *O. mungos*.

Application of research: This research has made an attempt to develop a protocol for the transient expression system for strictosidine synthase (CrSTR) in *O. mungos* and this technique could be of help in developing transgenics of *O. mungos* and overproduction of camptothecin.

Research Category: Crop Physiology

Abbreviations: *Catharanthus roseus* strictosidine synthase (CrSTR) and Camptothecin (CPT), β-glucuronidase(GUS).

Acknowledgement / Funding: Authors are thankful to University of Agricultural Sciences, GKVK, Bengaluru, 560 065, India

***Research Guide or Chairperson of research: Professor Umashaanker R.**
University: University of Agricultural Sciences, GKVK, Bengaluru, 560 065
Research project name or number: MSc Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript

Conflict of Interest: None declared

Sample Collection: Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bengaluru, 560065, Karnataka

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

References

[1] Wall M.E., Wani M.C., Cook C.E., Palmer K.H., McPhail A.T. and Sim G.A. (1966) *J. Am. Chem. Soc*., 88, 3888–3890.
[2] Govindachari T.R. and Viwanathan,N. (1972) *Phytochemistry*, 11, 3529-3531.
[3] Kulkarni A., Patwardhan A., Lele U. and Malpathak N. (2010) *Pharmacogn. Res*., 2, 296.
[4] Gunashekera S.P., Badawi M.M., Cordell G.A., Farnsworth N.R. and Chitnis M. (1979) *J Nat Prod*., 42, 475-477.
[5] Arisawa M., Gunasekera S.P, Cordell G.A and Farnsworth N.R. (1981) *Planta Med*., 43, 404-
[6] Zhou B.N., Hoch J.M., Johnson R.K., Mattern M.R., Eng W.K., Ma J., Hecht S.M., Newman D.J. and Kingston D.G. (2000) *J. Nat. Prod*., 63,1273–1276.
[7] Gharpure G., Chavan B., Lele U., Hastak A., Bhave A., Malpure N., Vasudeva R., Patwardhan A. (2010) *Curr. Sci*., 98, 302-304.
[8] Beegum A.S., Martin K.P., Zhang C.L., Nishitha I.K., Ligimol., Slater A. and Madhusoodanan P.V. (2007) *Electron J Biotechnol*., 10 (1), 114-123.
[9] Patwardhan A. (2006) *The rufford small grants foundation, UK*, pp. 7-15.
[10] López-Meyer M., Nessler C., McKnight T. (1994) *Planta Med*., 60, 558-560.
[11] Ramesha B., Amna T., Ravikanth G., Gunaga R.P., Vasudeva, R., Ganeshaiah K., Uma Shaanker R., Khajuria R., Puri S. and Qazi G. (2008) *J. Chromatogr. Sci*., 46, 362-368.
[12] Watase I., Sudo H., Yamazaki M. and Saito K. (2004) *Plant Biotech*., 21(5), 337-342.
[13] Rajan R., Varghese S.C., Kurup R., Gopalakrishnan R., Venkataraman R., Satheeshkumar K., Baby S. (2013) *Ind. Crops Prod*., 43, 472–476.
[14] Martin K., Beegum A.S., Zhang C.L., Slater A., Madhusoodanan P. (2007) *Biol. Plant*, 51, 769-772.
[15] Vanisree M. Lee C.Y., Lo S.F., Nalawade S.M., Lin C.Y. and Tsay H.S. (2004) *Bot. Bull. Acad. Sin*., 45,1-22.
[16] Sakato K., Tanaka H., Mukai N., Misawa M. (1974) *Agric. Biol.Chem*., 38, 217-218.
[17] Wiedenfeld H., Furmanowa M., Roeder E., Guzewska J., Gustowski W. (1997) *Plant Cell, Tissue Org. Cult*., 49, 213–218.
[18] Roja G. and Heble M. (1994) *Phytochemistry*, 36, 65–66.
[19] Ciddi V. and Shuler M. (2000) *Biotechnol. Lett*., 22, 129–132.
[20] Saito K., Sudo H., Yamazaki M., Koseki-Nakamura M., Kitajima M., Takayama H., Aimi N. (2001) *Plant Cell Rep*., 20, 267–271.
[21] Sudo H., Yamakawa T., Yamazaki M., Aimi N. and Saito K. (2002) *Biotechnol. Lett*., 24, 359–363.
[22] Lorence A. and Nessler C.L. (2004) *Phytochemistry*, 65, 2731–2741.
[23] Canel C. M., Cardoso I. L., Whitmer S., Leslie V. D. F., Pasquali G., Robert V. H., Harry J., Hoge C and Verpoorte R. (1997) *Planta*, 205, 414-419.
[24] Van der fits., and Memelink, J. (2000) *Science*, 289(5477):295-297.
[25] Karcher S. J. (2002) *Proceedings of the 23rd Workshop/Conference of the Association for Biology Laboratory Education*. 23, 29-42.
[26] Padmanabha B.V., Chandrashekar M., Ramesha B.T., Hombe G.H.C., Gunaga R.P., Suhas S., Vasudeva R., Ganeshaiah K.N. and Uma S.R. (2006) *Curr. Sci*., 90(1), 95-99.
[27] Yan X.F., Yang W., Tao Y., Yu-hong Z. and dai S.J. (2003) *Bot. Bull. Acad. Sin*., 44,99-105
[28] Gomez K.A. and Gomez A.A. (1976) *International Rice Research Institute, Los Banos, Philippines* 264.
[29] Kapila J., Rycke R., Montagu V. M. and Angenon G. (1997) *Plant Sci.,* 122, 101-108.
[30] Verpoorte R. and Memelink J. (2002) *Curr Opin Biotechnol*., 13, 181-187.
[31] Memelink J., Kijne J.W., Heijden V.D.R. and Verpoorte R. (2001) *Adv Biochem Eng Biotechnol*., 72, 103-25.
[32] Wang C., Liu H., Gao X. and Zhang H. (2012) *Plant Cell Rep*., 29, 887-894.
[33] Mehrotra S., Srivastava V., Rahman L.U. and Kukreja A.K. (2013) *Plant Cell Tiss. Org*., 115, 377-384.
[34] Cui L., Ni N., Qian J., Teng X., Yang Y., Chao W., Zekria D., Zhang D. and Kai G. (2015) *Sci. Rep*., 5, 8227.