EXPLOITATION OF *IN VITRO* INDUCED ZYGOTIC POLYEMBRYONY FOR GENETIC TRANSFORMATION IN *HEVEA BRASILIENSIS*

Rekha, K, Jayashree, R., Sushamakumari, S., Sobha, S Sagin, J and Thulaseedharan. A

Advanced centre for Molecular Biology and Biotechnology
Rubber Research Institute of India, Kottayam, 686009.India

ABSTRACT

Inspite of a few successful events in genetic manipulation, *Hevea* is still facing the problem of regeneration and hardening of transformants. Use of more juvenile explants may be a solution to address this issue. Multiple embryos /embryogenic calli derived from zygote as the target tissue have been used in many plants for the regeneration of transgenic plants (Dandekar1989). In *Hevea*, attempts were made to induce embryogenic calli from immature zygotic embryos by adopting *in ovulo* embryoculture method (Rekha, et.al.2010) coupled with media manipulations. Immature fruits were collected from field grown trees of *Hevea*, surface sterilized and cultured in the medium. Multiple embryos along with embryogenic calli were induced in Nitsh basal medium supplemented with the growth regulators of zeatin (0.3 mgl^{-1}) along with Gibberellic acid (2 mgl^{-1}) and kinetin (3 mgl^{-1}). Calli were proliferated and used as the target tissue for transformation experiments with three different gene constructs namely Osmotin, hmgr1 and MnSOD. High frequency transformation (70%) could be achieved. Transgenic cell lines were developed and proliferated from all the gene constructs. Successful embryo induction, maturation and plant regeneration were achieved with all the three different gene constructs. Plants have been developed and hardened. Compared to explants of clonal origin, plant regeneration was faster and easier for this explant. The time span for the regeneration of plants could be reduced to 1 year compared to clonal explants which usually takes 2-3 years. The percentage recovery of plants after hardening also could be improved considerably. Regeneration of plants from more events is possible since the system allows easy and rapid plant regeneration and hardening. This inturn enables more effective evaluation and the superior events among the transformants can be identified. Plants developed can be used as breeding materials in hybridization programme. Moreover, this system can be effectively utilized in functional evaluation of the foreign genes inserted. Development of transgenic rootstocks is another possibility of this technique.

Key words:in ovulo **embryo culture, zygotic polyembryony, genetic transformation**

INTRODUCTION

Recombinant DNA technology has become a powerful tool for crop improvement since it provides a means of genetic manipulation that can bypass sexual barriers and to some extent circumvent the limitations of the long breeding cycle of perennial trees. Over the past decade, the value of introducing foreign gene in to plants has been well documented. This technology could minimize difficulties associated with traditional breeding and reduce the time necessary to produce genetic changes in woody species like *Hevea.* The ability to obtain specific expression of foreign or native genes in *Hevea* opens up the possibility of improving this important crop commercially by genetic manipulation. A wide range of traits could be introduced without compromising the genetic back ground of the elite clones, provided a reliable method of plant regeneration from the transformed cell is available. While highly attractive in concept, with tremendous scientific potential, generation of a transgenic crop plant is confronted with innumerable technical hurdles. Technical problems involved in transformation and regeneration are much more severe and demanding in woody perennial species. *Hevea* represents one of the most difficult systems. Inspite of a few successful events in genetic manipulation, *Hevea* is still facing the problem of regeneration and hardening of transformants. Generally, prior to the development of transgenic plants, gene integration and effects of foreign gene insertion are studied using model systems like tobacco and *Arabidopsis*. In a perennial tree crop like *Hevea*, a better candidate for this purpose is the, multiple embryos /embryogenic calli derived from zygote, which are more juvenile and hence more responsive than clonal explants. By incorporating such juvenile tissue in transformation experiments, the functional validation of the integrated genes can be accelerated to a great extend. In the present experiment successful induction of embryogenic calli from immature zygotic embryos of *Hevea* and its potential in genetic transformation experiments is discussed.

MATERIALS AND METHODS

Explant generation

Immature fruits (8-10 wks old) were collected from field grown trees of *Hevea (*clone RRII 105*),* washed thoroughly in distilled water and allowed to dry. Fruits were then dipped in 80% alcohol for 15 minutes and allowed to dry on a sterile petriplate. The ovul were isolated from the fruits using sterile knife and inoculated in the nutrient media. Since embryos are not visible at early stages and are difficult to dissect, the ovules were cut into two halves and placed in the medium with the micropylar end touching the medium (Rekha, *et al.,* 2010). The basal medium used for the experiment was Nitsch (1960). The pH of the medium was adjusted to 5.7 with 1N KOH and solidified with 0.2% phytagel. The medium was autoclaved at 121^0C, 15 lb pressure for 10 minutes. The medium was poured into sterile disposable Petri dishes and wrapped with parafilm. The growth regulator combination of GA_3 (2 mgl^{-1}) and kinetin (3 mgl^{-1}) reported earlier for the induction of multiple embryos (Rekha et al 2010) was kept constant and different levels of other growth regulators viz. Zeatin, NAA, 2, 4-D and BA were tried along with them for the induction of embryogenic calli. Observations were recorded at weekly intervals.

Genetic transformation

The embryogenic calli obtained from this cultures were proliferated and used as the target tissue in transformation experiments with three different binary vectors the gene encoding osmotin protien (Barthakur,.*et al* 2001), hmgr1 (Hydroxy Methyl Glutaryl CoA Reductase) (Venketachalam *et al* 2009) and MnSOD (Manganese Superoxide Dismutase) (Jayashree,et al.,2003) . Transformation experiments were carried out as follows. Forty to

fifty micro liters of the glycerol stock of each construct was added to 5 ml MG/L medium (Jones *et al.*, 2005) with the respective antibiotics and the pH was adjusted to 7. The cultures were kept in an incubator shaker overnight at 28°C at 250 rpm. 2.5 ml of the overnight grown culture was transferred to 7.5 ml of Tryptone Yeast extract (TY) medium (Beringer, 1974) with pH 5.5 containing the antibiotics and 200 µM acetosyringone. The cultures were incubated overnight at 28°C at 250 rpm. On the third day 1.5 ml of the bacterial culture was diluted to 20 ml by adding TY medium (pH 5.5), containing acetosyringone (200 µM) and the respective antibiotics antibiotics. The OD was measured against TY blank at 600 nm and the concentration adjusted to get an optimum OD of 0.2. This bacterial culture was used for infecting the target tissue.

The friable embryogenic callus (2 gm) was kept in small petriplate and soaked with *Agrobacterium* suspension using a micropipette. After 5 minutes, the calli was blotted dry using a sterile filter paper and transferred to co-culture media. The co-culture media contains 200 µM acetosyringone, 115 mgl^{-1} proline and 113 mgl^{-1} glycine betaine along with the other ingredients in the embryogenic callus induction medium. Co- culture was done at 28° C for 3 days. After 3 days of co-culture, the infected calli were blotted dry on a sterile filter paper and transferred to selection medium containing 500 mgl^{-1} cefotaxime and 350 mgl^{-1} kanamycin for osmotin and SOD genes and 30 mgl^{-1} hygromycin for hmgr gene and maintained at 25±2° C in the dark. Sub culturing was done at three week interval. After two subcultures, cefotaxime was omitted from the medium and screening for putatively transgenic lines in the presence of kanamycin was continued. After 60 days of co-culture, observations were recorded. The putatively transgenic cell lines that emerged from these selection plates were separated individually and transferred to proliferation medium. Since each line represents a single transformation event, each transgenic line showing antibiotic resistance was handled individually.

Confirmation of gene integration

From the proliferated, putatively transgenic cell lines, five lines were randomly selected. DNA was isolated according to the standard protocol (Doyle & Doyle1990) and PCR analysis was performed using gene specific primers using the standard protocol (Sambrook *et.al* .1989).

RESULTS AND DISCUSSION

It was observed that among the different growth regulators tried, Zeatin in presence of GA_3 and Kin had a positive influence in the induction of multiple embryos. Multiple embryos were induced in Nitsch basal medium supplemented with the growth regulators zeatin (0.3 mgl^{-1}), GA_3 (2 mgl^{-1}) and kinetin (3 mgl^{-1}). The results are presented in table.1. There is a steady increase in the number of multiple embryos with increasing concentrations of growth regulators. A maximum of 37 embryos could be obtained in presence of the above growth regulator combination. In the same combination, along with multiple embryos, emergence of embryogenic calli also could be observed (Figure.1). Among the other three growth regulators tried, BA induced germination of the zygotic embryo and prevented the formation of multiple embryos. Both NAA and 2, 4-D induced callusing from the outer integument and inhibited the development of embryos inside the ovule. The influence of growth hormones in the development of polyembryonic seeds is described by many workers. Haccius (1955) demonstrated that in the undifferentiated embryos of *Eranthis hiemalis*, differentiation was transiently arrested in the presence of 2,4-D. In the present study also 2, 4-D has a negative influence on the induction of multiple embryos. The favorable effect of GA_3 and kinetin on the development of somatic embryos of *Hevea* was reported earlier

(Sushamakumari et al., 2000). Also positive influence of Kinetin and GA_3 on the growth of the zygotic embryo was reported earlier in *Hevea* (Rekha *et al.*, 2010). Kinetin and GA_3 were successfully used in embryoculture of cucumis melo for the rescue of young embryos (Alexander and Yigal (1994) .Induction of polyembryony and formation of embryonal masses in presence of kinetin is reported in fertilized ovules of *Ribes rubrum* L. cv. F. Hosszúfürtü. (Zatykó 1975) .Induction of polyembryony and development of multiple embryos and plantlets were reported in *Hevea* earlier. Molecular analysis of these plants revealed that they are of zygotic origin and the plants derived are true-to type (Rekha, *et.al.*2010).This phenomenon can be regarded as a type of cleavage polyembryony where cleavage of the original zygotic embryo may occur either at a few-celled stage or later in the phase of radicular meristem establishment.

Table 1.Effect of various growth regulators on the development of cultured ovules

Treatments	**G A_3 mgl^{-1}**	**Kin mgl^{-1}**	**zeatin mgl^{-1}**	Number of mean no of embryos obtained.
T1	3	2	0.1	13
T2	3	2	0.2	14
T3	3	2	0.3	37 ##
T4	3	2	0.4	12
T5	3	2	0.5	15
				CD (1%) 3.93

Embryos with embryogenic calli

Genetic transformation

It was observed that when embryogenic callus derived from zygotic embryo was used as the target tissue for genetic transformation, emergence of transgenic lines was faster. In this case putatively transformed cell lines could be obtained within 30 days after infection with *Agrobacterium,* whereas in the case of clonal material as target tissue, it took almost 60 days. The transformation frequency varied from 63-80% depending on the gene construct (Figure2). Highest transformation frequency (70%) was obtained with osmotin gene followed by 68% with SOD and 63% for hmgr1 gene. Transgenic cell lines from all the gene constructs were isolated and proliferated in MS basal medium supplemented with growth regulators BA, GA_3 and Kinetin. The gene integration was confirmed in all the randomly selected lines of each gene construct by PCR analysis (Figure.7,8 &9). The data on transgenic plant regeneration is presented in table.2. High frequency embryo induction (62-65%) was achieved with three different gene constructs (Figure.3&4). Plant regeneration has been achieved with 2 gene constructs and the frequency varied from 60-62 % (Figure.5). Plants have been developed and hardened (Figure.6). Compared to explants of clonal origin, plant regeneration was faster and easier for this explant. The survival rate during hardening is much higher compared to clonal material. The time span for the regeneration of plants could be reduced to

1 year compared to clonal explants which usually takes 2-3 years. The increased efficiency of embryogenic callus formation and regeneration of plantlets was observed in many crops including wheat by using polyembryos derived from the zygote as the explant. (Erdelska,*et al*,.1995). The secondary somatic embryos derived from the cotyledonary explants of zygotic embryos were successfully utilized for the development of transgenic plants in Walnut (Dandekar et.al., 1989). In clonally propagated woody plants the explant source is an important consideration in maintaining clonal integrity. Eventhough zygotic explants are less desirable for maintaining clonal integrity; this can be used as a model system for functional validation of inserted gene especially in recalcitrant woody tree crops.

Table 2. The transformation frequency and subsequent plant regeneration with different genes using *embryogenic callus derived from immature zygotic embryo as the target tissue.*

Gene constructs	Transformation frequency	Embryo induction	Maturation	Germination and plant conversion
Osmotin	70%	65%	80%	60%
Hmgr1	63%	60%	74%	62%
SOD	68%	62%	Awaited	Awaited

CONCLUSIONS

A system for the faster and easier development of transgenic plants has been developed in *Hevea brasiliensis* by using callus derived from immature zygotic embryos. Efficient transformation, embryo induction, maturation and plant regeneration could be achieved with different gene constructs. The percentage recovery of plants after hardening could also be improved considerably. Regeneration of plants from more events is possible since the system allows easy and rapid plant regeneration and hardening. This in turn enables more effective evaluation and identification of the superior events among the transformants. This system can be effectively utilized in functional validation of the integrated genes. Development of transgenic root stocks is another possibility of this technique.

ACKNOWLEDGEMENT

The authors wish to extend their gratitude to Dr. James Jacob, Director, Rubber Research Institute of India for his constant encouragement given during the study. They also wish to thank Mr.Aneesh, for the help rendered in statistical analysis.

REFERENCES

Alexander, B and Yiga,C (1994). Effect of kinetin and GA_3 on in vitro ovule embryo culture of *Cucumis melo* L., **16 (3)** 267-269

Barthakur, S., Babu, V. and Bensal, K.C. 2001.Over-expression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. J. Plant Biochem. Biotech. **10**: 31-37.

Beringer, J.E. 1974. R. factor transfer in Rhizobium leguminosarum. J. Gen. Microbiol. 84:188-198

Doyle, J. J. and Doyle, J. L. 1990. Isolation of DNA from small amount of plant tissues. Focus. 12: 13-15.

Haccius.B. 1955.Experimentally I nduced Twinning in plants.Nature.176(4477):355-356.

Jayashree, R., Rekha, K., Venkatachalam, P., Uratsu, S.L., Dandekar, A. M., Kumari Jayasree, P., Kala, R.G., Priya, P., Sushamakumari, S., Sobha, S., Asokan, M. P., Sethuraj, M.R. and Thulaseedharan, A. 2003. Genetic transformation and regeneration of rubber tree (*Hevea brasiliensis* Muell. Arg.) transgenic plants with a constitutive version of an anti-oxidative stress superoxide dismutase gene. Plant Cell Rep. 22:201-209

Jones, H.D., Doherty, A. and Huxia, W.U. 2005. Review of methodologies and a protocol for the *Agrobacterium* mediated transformation of wheat. Plant Methods. **1:** 5.

Nitsch, J.P (1969). Experimental androgenesis inNicotiana.Phytomorphology 19:389-404

Rekha, K., Jayashree, R., Gireesh, T., Sushamakumari, S., Jomini, T., Priya, .A.,Jayasree P. K., Sobha, S., Kala, R.G. and Thulaseedharan. A. (2010). Embryo rescue and plant regeneration in *Hevea brasiliensis.*Natural Rubber Research (In press)

Rekha, K., Jayashree, R., Sushamakumari, S., Sobha, S., Gireesh, T., Supriya, R., Anitha, A., Saha, T. and Thulaseedharan, A. (2010) Induction of zygotic polyembryony and induction of true-to-type seedlings of *Hevea brasiliensis. International Conference on "Biotechnology: A Global Scenario".* 2-4 November, Kakatiya Universit Warangal, India. *pp*: 121-122

Sambrook J., Fritsch, E.F. and Maniatis, T.1989. *Molecular cloning: a laboratory manual.* Second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NewYork.

Venkatachalam. P., Priya. P., Jayashree. R., Rekha. K. and Thulaseedharan. A. (2009). Molecular cloning and characterization of a 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (hmgr1) gene from rubber tree (*Hevea brasiliensis* Muell. Arg.): A key gene involved in isoprenoid biosynthesis. Physiol. Mol. Biol. Plants. 15:133-143

Zatykó, J.M I. and Szabó S,C. 1975. Induction of polyembryony in cultivated ovules of red currant Plant Science letters.4 (5): 281-283

Erdelska,O and Vidovencova,Z.,and Erdelsky1995.Cleavage polyembryos as explants for plant regeneration in wheat.1995.Plant cell reports,.15(5):342-344

Dandekar, A.M.,Granahan,G.H.,Leslie,C.A and Uratsu,S.L1989.*Agrobacterium* mediated transformation of somatic embryos as a method for the production of transgenic plants. Journal of tissue culture methods.12 (4):145-149

Sushamakumari,S.,Sobha,S.,Rekha,K.,Jayashree,R., AND Ashokan,M.P. (2000).Influence of growth regulators and sucrose on somatic embryogenesis and plant regeneration from immature inflorescence of Hevea brasiliensis. Indian journal of Natural Rubber Research, 13 (1&2): 19-29.

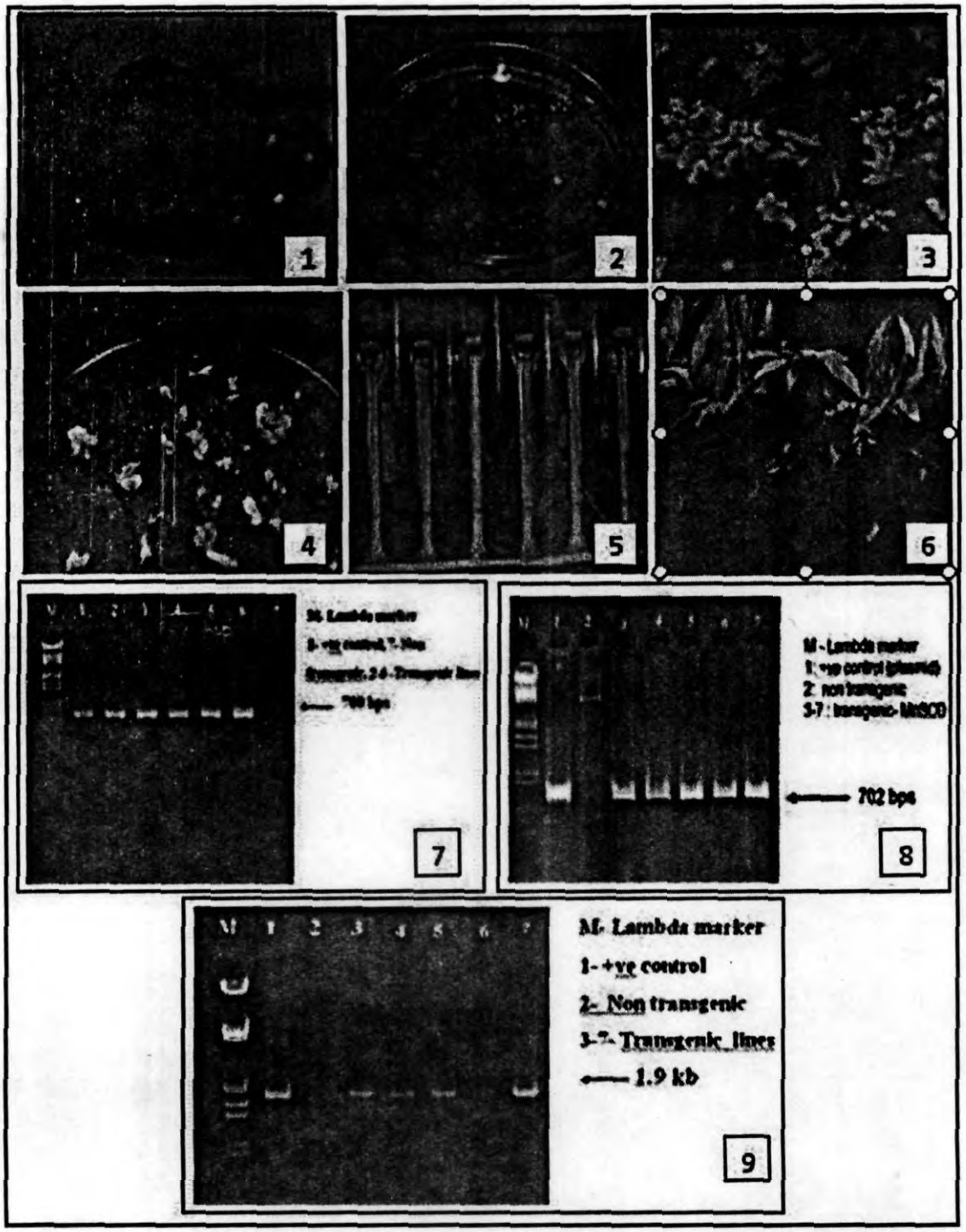

Figure 1. Emergence of polyembryos with embryogenic calli
Figure 2. Emergence of transgenic cell lines
Figure 3. Embryo induction
Figure 4. Embryo maturation
Figure 5 Plant regeneration
Figure 6. Hardened plants
Figure 7. PCR Analysis of transgenic cell lines integrated with osmotin gene.
Figure 8. PCR analysis of transgenic cell lines integrated with SOD gene.
Figure 9. PCR analysis of transgenic cell lines integrated with hmgr1 gene.