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Research paper

Efficacy analysis of various target tissues for genetic transformation to overcome recalcitrance in perennial tree crops

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Abstract

Recalcitrance and low regeneration rate of perennial tree crops are the major hurdles faced by biotechnologist working on the development of transgenic tree varieties around the world. The nature of target tissue is a major deciding factor which determines the success in various stages of transgenic development like transformation, plant regeneration and hardening. *Heveabrasiliensis,* the major source of natural rubber is a perennial tree crop and the aforementioned problems have slow downed the efforts to developtransgenic *Hevea*varities with better agronomic traits. To overcome these issues in *Hevea,* an attempt was made in this study to compare theefficiencies of different somatic and zygotic explants starting from the tissue infection stage to plantregeneration and hardening.The results shows that compared to embryogeniccalli from anther and ovule a high transformation frequencyof 76.40%was obtained from embryonic calli derived from zygotic embryo. A clear advantage of zygotic explants wasapparent in different stages of plant regeneration and hardening also. Moreover, the embryos derived from this source had more number of lateralroots andshowed bettervigour than clonal explant. The results from our study indicate that, zygote derived target tissue are much better than somatic derived calli to overcome the problems associated with recalcitrance and lower regeneration frequencies often observed among transgenic perennial tree crops.

Key words: *Heveabrasiliensis*; genetic transformation; clonal explants; recalcitrancy

Introduction

Genetic transformation of plants is an innovative research tool which has practical significance for the development of new and improved genotypes or cultivars and also to gain information about gene function. The stable introduction of genes of interest into nuclear genomes depends on several factors such as the choice of target tissue, the method of DNA delivery in the target tissue, and the appropriate method to select the transformed plants. Over the past few years several protocols have been developed for both angiosperms and gymnosperms which utilize biological and non-biological methods for gene integration. Despite great advancements in this area, there are still several aspects of plant transformation that need to be elucidated. One among themis the ability to regenerate viable plants from

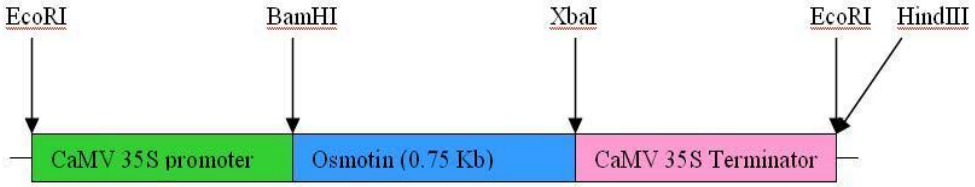
the transformed cells. The advancements in tissue culture practices which have occurred over the past decades could not contribute much towards the rapid and efficient regeneration of transformants, especially in recalcitranttree crops.

Heveabrasiliensis (Para rubber) belonging to the family *Euphorbiaceae*, is the major source of natural rubber for commercial utility and it accounts for 99 percent of the world's natural rubber. Lesser tolerance of *Hevea*to heat, drought and cold conditions is the main factors limiting its planting in non-traditional areas of India. Even though rubber is grown in some of the NE states in India, the productivity of these plantations are very less compared to plants grown in the traditional rubber growing regions. This is mainly due to the cold stress encountered by the plants at NE. Similarly, high temperature and drought conditions limits the cultivation of rubber in central India especially in states like Maharastra, Orissa etc. Therefore, development of rubber clones with increased tolerance to the above stress is imperative to extend rubber cultivation to these areas. *Heveabrasiliensis*, being a perennial tree crop, development of transgenics is a cumbersome process. Despite the development of few transgenics, regeneration of transgenic tissues into viable plants still remains as an uphill task, due to several constraints particularly difficulties associated with generation of ideal target tissues ,low plantlet conversion ability and finally hardening of valuable regenerants. In the present study, we have tried genetic transformation of *Heveabrasiliensis* using the gene coding for osmotin protein under the control of CaMV35S promoter with different explants and their performance is compared until plant regeneration and hardening. Since osmotin plays a crucial role in alleviating the impact of stress in plants, over expression of this genes in *Hevea* by transgenic approach may confer the plant with better cold and drought tolerance properties. The study was attempted with7different explants initially with the intention of developing the right transformation strategy for a tree crop like *Hevea* where recalcitrance rates are very high.

Materials and methods

Binary vector used for transformation

Agrobacterium strain GV 2260 harbouring the plasmid osm/BinAR under the control of CaMV35 S promoter containing kanamycin resistance as the selectable marker (Barthakur*et al.*, 2001) was employed for genetic transformation (Fig.1).

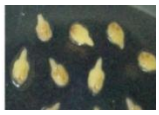
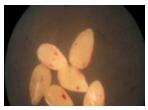
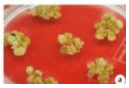


Target tissues

Seven different target tissues derived from three explant sources including both clonal and zygotic origin was used (Table 1).Immature inflorescence were collected from field grown trees of the clone RRII105.After surface sterilization, immature anthers at diploid stage (before microsporogenesis) were dissected out aseptically under a stereomicroscope from

the male flower buds and inoculated for the induction of primary callus followed by embryogeniccalli as per the described protocol (Jayasree*et al.,* 1999). Theimmature ovulesbefore megasporogenesis were dissected out from the female flowers and cultured for primary andembryogeniccalli (Jayashree*et al* .,2011). Similarly immature fruits were collected from field grown plants and after through washing and surface sterilization,zygotic embryos were cultured according to half *ovulo* embryoculture technique (Rekha*et al.,* 2012). The intact explants, primary calli and embryogeniccalli from all sources were used as target tissues for transformation.Among the different target tissues attempted for transformation, only the most responsive ones namely embryogeniccalli from anther as well as embryogeniccalli from zygotic embryo were used for subsequent experiments. Optimization of different stages of somatic embryogenesis for transgenic plant regeneration were done using the clonal explant anther derived embryogeniccalli.

Table. 1Different target tissues used for the integration of osmotin gene transfer in *H. brasiliensis*

Source	Explant	Target tissues
Clonal	A. Immature anther 	Intact anther Primary calli  Embryogeniccalli 
	A. Immature ovule 	Intact ovule Primary calli  Embryogeniccalli 
Zygotic	Immature zygotic embryo	Embryogeniccalli 

Procedure used for transformation

To perform genetic transformation, two different protocols as reported by Jayashree*et al.*, (2003)and Rekha*et al.*(2010) was tried.

The details of the two protocols are summarized in Table.2

Table.2 Protocols followed for *Agrobacterium* mediated transformation

Steps followed	Method-1	Method-2
Inoculum preparation	4 days procedure	3 days procedure\
Temperature at which bacteria was grown	28^0C	23^0C
Bacterial density	$5x10^8$ cells/ml	$5x10^8$cells/ml
Optimum O.D	0.5 at 420nm	0.1-0.2 at 600nm
Inoculation medium	*AELB followed by MS Acetosyringone (20 mg l^{-1})	*MGL followed by TY Acetosyringone (40 mg l^{-1})
Co-culture medium	Solid Acetosyringone (20 mg l^{-1})	Liquid Acetosyringone (40 mg l^{-1})
Co-culture temperature	28^0C	23^0C

Selection of transgenic cell lines and proliferation

After the co- culture period, infected tissues were transferred to media designed for the inhibition of the growth of *Agrobacterium* as well as selection of transformants. In *Hevea,* cefotaxime has been reported to be an ideal antibiotic for suppressing *Agrobacterium*. The *npt11* gene imparting kanamycin resistance is incorporated in the gene construct along with the gene of interest. The concentration of kanamycin for effective selection was based on methods standardized previously (Jayashree*et al.*, 2003; Sobha*et al.*, 2003; Rekha*et al.*, 2006; Kala *et al.*, 2006). After 3 days of co culture, the infected calli were blotted dry using a sterile filter paper and were transferred to selection medium containing 500 mg l^{-1}cefotaxime and 300 mg l^{-1} kanamycin and maintained at 25±2^{o}C in the dark and subculturing was done at three weeks interval.

After 30 days, cefotaxim was omitted from the medium and selection for putative transgenic cell lines in the presence of kanamycin was continued. The cultures were observed weekly and those cultures with bacterial overgrowth were discarded and the rest of the cultures were carried forward. Putatively transformed cell lines emerging from these cultures were selected and transferred to proliferation medium (Rekha*et al.*, 2013).

Confirmation of the transgene integration by PCR analysis

Osmotin gene integration was tested randomlyin selected cell lines developed from independent transformation events by PCR analysis using osmotin gene specific primers as per the standard procedure.

Development of transgenic plants

Embryo induction, maturation and plant regeneration were attempted in a previously standardized media (Rekha*et al.*,2013, 2014).

Comparison of clonal transgenic cell lines with that of zygotic origin was attempted under optimized media combinations.Twenty-five calli groups were used for embryo induction and the experiments were repeated 5 times. Observations on embryo induction were recorded after two months and percentage of embryo induction was worked out.For maturation, the same basal medium used for embryo induction, devoid of growth regulators other than ABA were used.Along with ABA, highphytagel and sugar was also added as per Rekha*et al.(2013)*.Fiftyembryos were kept per treatment and the treatments were replicated thrice. The mature somatic embryos with well-developed cotyledons were transferred to the germination medium. Poorly developed and abnormal embryos were discarded and MS medium, supplemented with different proportions of growth regulators BA and, GA_3 (0.5-5 mg l^{-1}) were tried for germination. After transferring to this media, embryos combinations were incubated in the dark condition for a period of 2 weeks and then transferred to light for shoot development.Experiments were repeated 5 times with 20 mature embryos per set.The bipolar differentiated embryos were transferred to plant regeneration medium (Jayasree*et al*., 1999).The pH of all media was adjusted to 5.7 prior to autoclaving at 121°C for 10 minutes. Media were solidified with 0.2% phytagel unless and otherwise mentioned. Cultures were raised in culture tubes containing 20 ml medium and incubated at 25 ± 2° C under cool white fluorescent lamps. Subculture was done every 3 weeks unless otherwise mentioned.

Acclimatization and molecular confirmation of transgenic plants

Plantlets after complete development and medium leaf maturation were carefully removed from the culture tubes and washed gently in running tap water to remove adhering medium. Dead tissues, if any, near the cotyledons were also removed. Plantlets were dipped in fungicide solution for 3 minutes in order to reduce fungal infection and were blotted to remove the adhering water particles by keeping for 2 minutes in tissue paper. Then they were planted in earthenware pots (5cmdiameter x 8 cm height) containing sterile sand, soil and soil-rite mixture and kept in the controlled conditions in growth chamber with a RH 90% and temperature 25°C . Humidity was decreased by 2 units at 2 days interval. Simultaneously, temperature was gradually increased from 25°C to 30°C. These plants were supplied with dilute Hoagland mixture weekly once and watering was done at two days interval. After about three weeks, the surviving plants were transferred to polybags filled with garden mixture (1:1:1) and kept in the growth chamber itself. After the emergence and maturation of a new flush, the hardened plants were taken out from the growth chamber and maintained in the shade house. These plants were irrigated at two days interval. NPK Mg (20:20: 0; 15) mixture was applied fortnightly. Transgene integration was confirmed by Southern hybridization and gene expression was estimated by reverse transcription PCR.

Results and discussion

Selection of putative transgenic cell lines

After 2 to 3 weeks of culture in the fresh medium with kanamycin, putative transformed cell lines, and showing resistance to kanamycin were emerged.Since each line represents a single transformation event, the putative transgenic lines were separated individually and transferred to proliferation medium fortified with kanamycin

Influence of target tissue on transformation frequency

Explants have a significant role in transformation efficiency as they behaved differently (Table 3). Among the different types of explants used for transformation, highest transformation frequency (76.4%) was obtained for the embryogeniccalli derived from immature zygotic embryo, followed by embryogeniccalli derived from anther (48%). Transformation frequency was very low for the two-month-old primary calli from anther. The intact explants as well as 2-month-old calli from the ovule failed to give any positive results and dried after two weeks in the selection medium. It was clearly indicated that embryogeniccalli produced higher number of transformants compared to primary calli. It was also observed that further proliferation of transgenic lines was better for embryogeniccalli, irrespective of the source. Even though a few transgenic lines were obtained from anther derived two-month-old calli, they failed to proliferate further.

Table.3 Influence of target tissue on transformation efficiency

No.	Explants	Transformation frequency Method 1	Method 2
1.	2 month old anther calli	6.00	11.60
2.	Embryogeniccalli from ovule	10.4	19.20
3.	Embryogeniccalli from anther	32.2	48.00
4.	Embryogeniccalli from zygotic embryo	44.8	76.40
	CD (0.05)	6.11	5.88

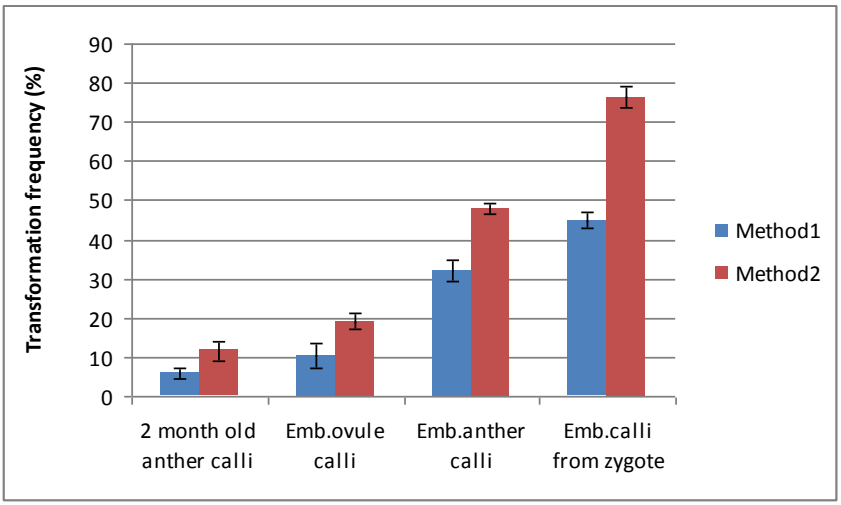
The availability of totipotent cells as targets for transformation is today the limiting factor in genetic transformation of recalcitrant woody species (Birch, 1997).Among the clonal explants, maximum number of transgenic lines could be obtained from embryogeniccalli derived from anther (48%). A higher transformation frequency of 76% could be obtained for embryogeniccalli derived from zygotic embryos. A few transgenic cell lines were obtained from 2 month old fresh callus derived from anther whereas 2 month old calli from ovule failed to give any positive results on transformation. This indicates that the initial source of the callus has a definite role in the frequency of transformation. Variation in the potential for different explants on transformation, somatic embryogenesis and organogenesis is well established (Piqueras*et al.*, 2010). Juvenility of the explant, friability and texture of the calli are the factors influencing transformation frequency. In Citrus, it has been reported that juvenile tissues showed higher sensitivity to *Agrobacterium* than the mature one (Cervera*et al.*, 2004). In cassava, *in vitro* induced shoots and germinating somatic embryos were used for transformation and highly variable plant regeneration efficiency (5–70%) was observed (Siritunga*et al.*, 2004; Puonti-Kaerlas*et al.*, 1997). Type of explant influenced transformation efficiency in monocots like wheat also (Lenglz*et al.*, 2009). Sarker and Biswas (2002) tested four different explants for their transformation ability in wheat and obtained maximum

transformation from immature embryo derived calli. It is generally observed that the texture of the callus and friability vary with initial explants and this will be reflected in the transformation and regeneration frequencies. The high transformation efficiency observed in the present study may be attributed to the juvenility, proliferation potential and fine texture of the callus derived from zygotic embryos. Similarly, embryogeniccalli are more friable and have higher proliferation potential compared to two month old primary calli. Embryogeniccalli has been used as a potential target tissue for genetic transformation in *Hevea*with different gene constructs (Rekha*et al*., 2006, Kala *et al*., 2006 Montero *et al*., 2003,Blanc *et al*., 2006) and high frequency transformation has been reported. The low transformation frequency observed in the present study for the two month old primary calli may be due to the lack of friability of the callus. The absence of dedifferentiation and lack of penetration of the *Agrobacterium* may be the reasons for the failure of intact explants to give any positive results. From the present study, it can be inferred that embryogeniccalli derived from anther as well as embryogeniccalli from zygotic embryo are the potential target tissues for obtaining successful transformation in *Hevea.* Use of embryogeniccalli as the target tissue has another advantage that it can considerably reduce the time span for transgenic plant regeneration, since it can bypass one crucial and most difficult step of the somatic embryogenesis pathway of *Hevea*namely induction of embryogeniccalli. In the case of zygotic embryo derived explants, more regeneration potential also is expected. Embryos and embryogeniccalli derived from embryos are being used in many crops for somatic embryogenesis (Gupta and Grob 1995; Klimaszewska and Cyr 2002; Von Arnold *et al*.,2002). However, after regenerating plants from the transgenic cell lines, a conclusion on explant selection can be arrived at.

Genetic transformation protocol

Among the two methods of transformation tried, method 2 was found to be ideal than the method 1 in three aspects. The transformation frequencies were significantly higher in method 2 compared to that of method 1 irrespective of the explants used. Number of transgenic lines could be enhanced from 32.2 to 48 % and 44.8 to76.4% for the anther derived and zygote derivedembryogeniccalli respectively.Also, emergence of transgenic cell lines from the cultures in the second protocol with liquid co-culture system was faster than the solid co-culture system. This may be due to the increased access of components including nutrients from the liquid co-culture medium.The results are given in Table.3& Fig.2

Fig.2Transformation frequency with different explants with 2 protocols



Embryogenesis and plant regeneration

Plantlets could be developed from transgenic cell lines belonging to the two different explant sources *viz*.embryogeniccalli derived from anther and embryogeniccalli derived from zygote, and are shown in **Fig.3**

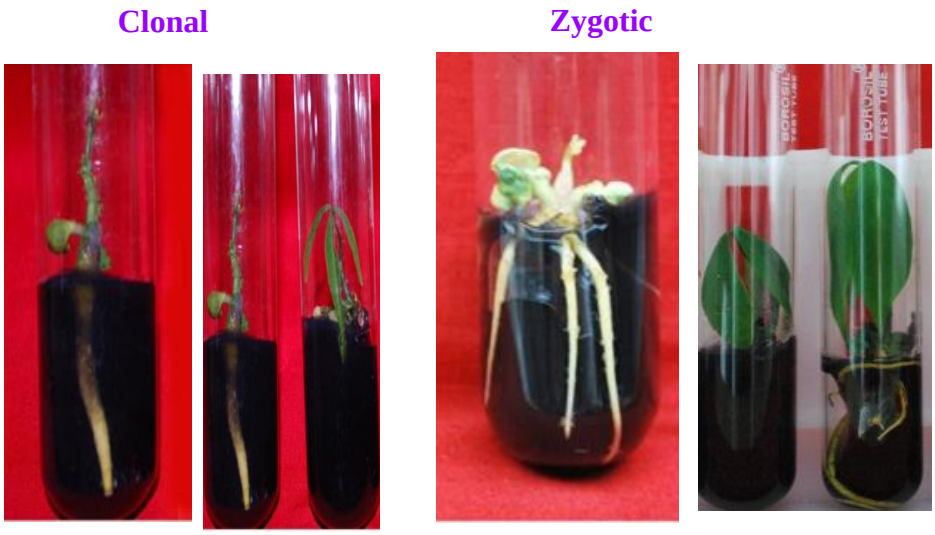
Table.4Efficiencies of transgenic cell lines derived from clonal and zygotic sources on different stages of the regeneration pathway

No	Embryo induction (%)	Embryo maturation (%)	Embryo germination (%)	Plant conversion (%)	Hardening (%)
Clonal	67	62	27	23	10
Zygotic	72	65	44	40	50

It was observed that the initial source of target tissue had a strong influence on plant regeneration from transgenic cell lines. During the course of plant development, the highest response was registered for zygotic embryo derived callus, compared to immature anther derived callus which is clonal in origin. For embryo induction and maturation, no significant difference was noticed between the cell lines. However, the embryos were larger and with well-developed cotyledons in the case of zygotic embryo derived transgenic callus. Also, plant conversion frequency for zygotic embryo derived transgenic embryos was much higher (almost double) compared to embryos of clonal origin. The plant conversion frequency of 40% was obtained from zygotic embryo derived transgenic cell lines, whereas anther derived transgenic cell lines showed only around 23% conversion frequency (Table 4).

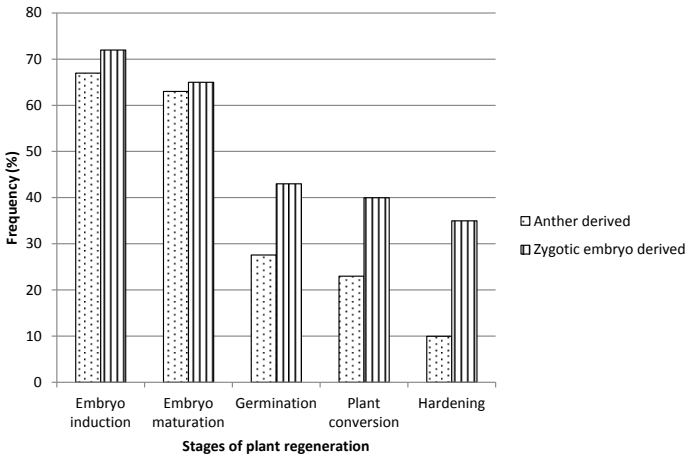
It was also observed that the plants derived from zygotic source responded favorably to acclimatization. They are healthier and had more number of lateral roots compared to anther derived plants (Fig.3).

Fig.3 Germination and plantregeneration from transgeniccell lines from different sources



Thirty-five transgenic plants could be regenerated from the clonal material. However, upon acclimatization, the mortality rate was high leaving behind only 10 plants at the end of initial hardening in the growth chamber and they were transferred to big polybags. Later on, after 2 months two surviving plants could be transferred to the net house. On the contrary, out of the 16 transgenic plants raised from the zygotic source, eight plants could be transferred to the net house.

Fig.4 Comparison of clonal and zygotic transgenic cell lines on different stages of plant regeneration



In the present study, plant regeneration could be achieved from 2 different explants raised from two different sources *viz*. Anther and zygotic embryo. A notable difference in

the frequencies of embryo induction, maturation germination and plant regeneration was observed in the presently developed media (Fig.4). The frequencies of germination, plant regeneration and hardening are very low for clonal explants compared to zygotic embryo derived transgenic lines. As a general rule, juvenile tissues, such as zygotic embryos, have better potential and competence to produce embryos and organs compared to more differentiated and mature tissues. Zygotic embryo has been used as explant for somatic embryogenesis and for developing transgenic plants in different crop species (Elhiti and Stasolla2011). 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. The secondary somatic embryos derived from the cotyledonary explants of zygotic embryos were successfully utilized for the development of transgenic plants in Walnut (Dandekar*etal*., 1989). In clonally propagated woody plants, the explant source is an important consideration in maintaining clonal integrity. Use of zygote derived explants implies a passage through sexual stage and therefore a drastic reshuffling of the genome which results in a subsequent alteration of clonal properties. 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. Recalcitrance of clonal explants prevents the production of transgenic plants on a commercial scale. Maturation and aging seem to be responsible for the decline in regenerative potential in woody plant species. Hence, use of zygotic material is a viable option for transgenic plant development. The majority of structural and physiological features needed for inducing somatic embryogenesis and organogenesis in culture are present in zygotic embryos. Zygotic embryo cells already express the "embryogenic potential" with many of the genes required for the induction process already expressed. Therefore, their fate is already committed and does not need to be redirected towards a new developmental path. This is why in many species embryogenic tissue can be readily obtained using immature or mature zygotic embryos. Degree of response in culture is also related to the developmental stage of the zygotic embryos. As a general rule, immature (early cotyledonary) embryos are more responsive than their fully mature counterparts. In the case of transgenics which is more difficult to regenerate, all these advantages of zygotic embryos could be well exploited. 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. In *Hevea* there is an added advantage of using these plants as stress tolerant root stocks and thus can bypass the biosafety concerns. However, embryogenic competence is often restricted to a short, yearly time window and therefore, identification of the stage of zygotic embryo to initiate an embryogenic line is of paramount importance.

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Author contribution

All the authors contributed equally to the work

Conflict of interest

Authors declares no conflict of interest

Compliance with Ethical Standards

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors

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