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EFFECT OF DIFFERENT PARAMETERS ON AGROBACTERIUM MEDIATED TRANSFORMATION IN *GLYCINE MAX*

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ABSTRACT
Agrobacterium tumefaciens has been an invaluable system in studying the fundamental biology of host pathogen interaction and plant biotechnology due to its unique ability to transfer DNA into the plant genome. It is widely used for transformation in dicots and later on used some monocots. *Agrobacterium* mediated transformation under laboratory condition is greatly influence by several factors such as infection time, source of explants, thiol compounds and acetosyringone. Standardization of parameters will helpful to transfer the agronomically important traits for improvement in present soybean cultivar. Some of these factors screened in soybean cultivar JS95-60. Soybean half seed explants of mature and premature seeds used. The selection of transformation carried out using expression of GUS reporter gene. The presence of acetosyringone in co-cultivation medium and the infection time of 4 hrs significantly enhanced the transformation frequency in mature and premature cotyledons. The pre mature cotyledons significantly responded as compare to the mature cotyledon upon agroinfection. 1.5 mM concentration of DTT at infection time of 4 hrs found to be optimum for maximum transformation efficiency which was about 13.33 percent. Similarly, co-cultivation medium supplemented with 4 mM cysteine increased the transformation efficiency (14.89 per cent). Our results demonstrate that this transgenic approach could be efficiently used to improve soybean quality and productivity through functional genomics.

KEY WORDS: *Agrobacterium tumefaciens*, JS95-60, GUS, DTT, cysteine, co-cultivation.

INTRODUCTION

Over the last 30 years, the field of genetic engineering has developed rapidly due to the greater understanding of deoxyribonucleic acid (DNA) as the double helix code from which genes are made. The term genetic engineering is used to describe the process by which the genetic makeup of an organism can be altered using recombinant DNA technology. A number of genetic modifications have been carried out in plants, animals and microbes since the inception of genetic engineering tools. Developing plant varieties expressing good agronomic characteristics is the ultimate goal of plant breeders. With conventional plant breeding, however, there is little chance of obtaining any particular gene combination from the millions of crosses generated. Undesirable genes can be transferred along with desirable genes; or, while one agronomic trait is gained, another is lost because the traits of both parents are mixed together and re-assorted more or less randomly in the offspring. These problems limit the improvements that plant breeders can achieve.

In contrast, genetic transformation offers the sources of foreign genes for plant improvement and strategies for over expressing or suppressing endogenous genes. Thus, introducing new traits or manipulating endogenous gene expression via transformation generates new phenotypic variation useful for investigating gene function and for crop improvement (Vignesh *et al*., 2010). Significant development has been done in the new and efficient transformation methods in plants like Biolistic gun, *Agrobacterium* mediated transformation, electroporation *etc*. Among these, biolistic and *Agrobacterium* mediated transformation are two predominantly used methods for insertion of a foreign DNA into host plant (Eady *et al*., 1996).

Agrobacterium tumefaciens is a soil bacterium which infects a wide range of dicotyledonous plant species and is capable of transferring a piece of DNA, located between the T-DNA borders of the Ti plasmid (T-DNA) into the genome of host plants, which makes *Agrobacterium* a natural genetic engineer (Gelwin, 2003). The transfer involves only the DNA between the T-DNA borders and some flanking sequences. Thus by deleting the wild-type crown-gall inducing T-DNA encoding genes and replacing it with selectable markers and other genes of interest "disarmed plasmid vectors" can be used to transfer foreign genes without disturbing the endogenous hormone balance of the infection. Simple co-cultivation techniques using explants such as leaf discs and selectable marker gene systems such as those encoding for kanamycin resistance (Atif *et al*., 2013) have become available and are being used extensively in gene transfer into higher plants. This method has been used successfully for transformation of numerous dicotyledonous species such as cotton, potato, tomato and soybean. More recently, *Agrobacterium*-

mediated gene transfer method has also been used successfully to transform agronomically important monocots like maize, wheat and rice (Kahariri, 2011).

The soybean (*Glycine max*) is a species of legume native to East Asia, widely grown for its edible bean that has numerous uses. The plant is classed as an oilseed rather than a pulse by the UN Food and Agricultural Organization (FAO) and is known as the "*GOLDEN BEAN"* of the 20th Century. However, due to presence of trypsin inhibitor and poor cooking ability, it cannot be utilized as a pulse. It is now the second largest oilseed in India after groundnut. It grows in varied agro-climatic conditions. It has emerged as one of the important commercial crop in many countries. Soybean has great potential as nutritive and very rich protein food. It can supply the much needed protein to human diets, because it contains above 40 % protein of superior quality and all the essential amino acids particularly glycine, tryptophan and lysine (5 %). Soybean also contains about 20 % oil with an important fatty acid, lecithin and Vitamin A and Vitamin D. The 4 per cent mineral salts of soybeans are fairly rich in phosphorous and calcium (Radhakrishnan and Kumari, 2009).

Though soybean is the major oilseed of the nation, the current level of its production has to be sustained and increased. As there are limits to area expansion, the production has to increase through yield increase. Lack of irrigation to this crop seems to be one of the main constraints in increasing its production. So improvement in soybean is necessary and thus introducing new genes or manipulating endogenous gene expression via soybean transformation is required to get a new phenotypic with improved trait (Kajale and Shroff, 2013). *Agrobacterium*-mediated transformation has several advantages over other methods of foreign gene introduction including its straight forward methodology, familiarity to researchers, minimal equipment cost and reliable insertion of a single transgene, or a low copy number (Yamada *et al*., 2012). This transformation method developed for gene introduction about fifty years ago and is still one of the most effective methods for introducing DNA into the nucleus of the target cells (King, 2013).

Agrobacterium mediated soybean transformation has several advantages over other methods of transformation. Advancement in soybean transformation appears to be slow in some of the local varieties of soybean. Reported *Agrobacterium* mediated transformation efficiencies are generally low and the protocols are often genotype specific. Transformation frequencies ranges from 0.2 % to around 10% and indicating that the transformation efficiency still relies on the skill of the practitioner and on the soybean genotype (Paz *et al.*, 2006). Increased soybean transformation efficiency may be achieved by further optimizing some culture conditions to enhance regeneration of transformed plants.

I. MATERIALS & METHODS

Establishment of *Agrobacterium* culture:

The *Agrobacterium* strain LBA4404 with construct pCAMBIA1301 grown on YEB agar containing 50mg/L kanamycin at 28˚C for two days. Single colonies of *Agrobacterium* obtained from plate and inoculated into 2 mL of YEB liquid medium containing 50 mg/L kanamycin for 8 hours at 28˚C, 250 rpm. Then 200 µL of this is inoculated to 200 ml of YEB and grown overnight to OD_{620} = 0.8 to 1.0 at 28˚C, 250 rpm, using a shaker incubator. Then the bacterial pellet collected by spinning the culture at 5000 rpm for 10 min and resuspended in infection medium for 1hr prior to the agro-infection. Glycerol stocks of 12% of overnight grown *Agrobacterium* culture to OD_{620} = 0.8 to 1.0 were prepared and maintained at -50˚C in a refrigerator. Bacteria cultures for weekly experiments are initiated from -50˚C glycerol stocks three days prior to an experiment.

Plant materials

a. Mature Seeds

Mature seeds of soybean [*Glycine max* (L.) Merrill], of cultivar JS 9560 were procured for project work wherever required. Soybean seeds were washed thoroughly under running tap water for three times. The seeds were then treated with a solution of the savlon (1% v/v) for 10 min and then surface sterilized with $HgCl_2$ (0.1% w/v) for 10 min under aseptic conditions. The seeds washed three times with sterile distilled water to remove excess $HgCl_2$. Under the laminar flow hood, approximately 18 hours prior to infection experiment, the deionized water added to the seeds and the bottle kept in dark.

b. Premature Seeds (pods)

Premature seeds of soybean [*Glycine max* (L.) Merrill], of cultivar JS 9560 were collected from experimental field during December to March. Soybean pods were washed thoroughly under running tap water for 3 times. The pods were then treated with a solution of the savlon (1% v/v) for 10 min and then surface sterilized with $HgCl_2$ (0.1 per cent w/v) for 10 min under aseptic conditions. The Pods washed three times with sterile distilled water to remove excess $HgCl_2$.

Explants preparation and infection

The premature seeds removed from soybean pods on a sterile paper towel for dissection. Using a scalpel blade, a longitudinal cut made along the hilum to separate cotyledons and to make small injury for infection of *Agrobacterium.* The seed coats removed with the help of pair of forceps. The seed divided into two parts *i.e.* half seeds. About 50 half seed explants were infected/immersed in *Agrobacterium* suspension culture *i.e.* infection medium for relative time.

Co-cultivation (CCM)

After infection, explants placed on sterile paper towel to drain excess liquid. Explants were then transferred adaxial side down on co-cultivation medium lined with sterile filter paper. Co-cultivation carried out for 7 days at 24˚C at 18:6 photoperiod.

Selection and Plant regeneration

After co-cultivation, the explants washed with the washing medium containing 100 mg/l cefotaxime antibiotic and then blotted on sterile blotting paper. The explants were cultured on solidified shoot induction medium (SIM I) with the cotyledon placed within the medium to stimulate shoot induction for the first 14 days and incubated at temperature of 25 ±2˚C under fluorescent lighting (90-150 µmol photons m2 s-1) in 18/6 h light.

After 1-2 subcultures, the regenerated shoots placed on fresh SIM II containing hygromycin B as selective agent.

After selection, shoots were sub-cultured to fresh SIM and incubated for another 14 days. The developed shoots were then transferred on to shoot elongation medium (SEM) for maximum elongation of shoots. The elongated shoots and/or leaves excised and further used for determination of transformation events by GUS assay.

GUS assay for confirmation of transformants

GUS assay performed in leaves and half-seed explants with regenerated shoots after 40 days of culture to check the stable GUS expression in shoots. All the plant materials and tissue sections washed with phosphate buffer containing 1 per cent (v/v) Triton X-100 and incubated for 1 hr at 37° C. The wash buffer replaced with 1mM X-Glue staining solution and the plant materials subjected to vacuum infiltration for 5 minutes. The plant materials then incubated in a 37° C incubator for 16-24 hrs.

Chlorophyll clearing

The X-Glue solution removed and the plant materials washed with double distilled water followed by wash with 70 per cent ethanol. To clear chlorophyll, the mixture of acetone: methanol (1:3) added and incubated at 4^0 C for 1h. This step repeated if necessary. After tissue became clear, washed with double distilled water twice and stored in 50 per cent glycerol.

II. RESULTS AND DISCUSSION

Effect of acetosyringone, infection time on transformation in mature and premature cotyledon

Several parameters known to influence the transformation efficiency of *Glycine max* evaluated to determine the optimal conditions for transformation. All the parameters were analysed based on transient gene β–glucuronidase (GUS) expression of the cotyledonary node of soybean explants. The effect of acetosyringone observed in mature and premature cotyledon on soybean and it observed that the premature cotyledons had a better efficiency of transformation as compare to mature cotyledons. Half seed explants were inoculated with *Agrobacterium* suspension for various times (1, 2, 3 and 4 hrs) to optimize transformation efficiency. After infection, explants co-cultured with *Agrobacterium* on CCM for 7 days in darkness. The results showed that the transient expression frequency of GUS gene increased with inoculation time. The highest frequency of transformants after GUS selection obtained when the premature half seed explants inoculated with *Agrobacterium* for up to 4 hours (12.76 per cent). In the mature cotyledon, 2 per cent, 2 % and 4 per cent transformation efficiency recorded after 2, 3 and 4 hour of the infection time respectively. The pre mature cotyledons showed about 3 times more transformation efficiency as compare to the mature cotyledons. Thus, the seed collected just before maturation given better response as compare to the mature cotyledons. The effect of acetosyringone on the transformation process also observed in mature as well as premature cotyledon. It was observed that the regenerated plants without acetosyringone did not show any positive confirmation after GUS analysis while both mature and premature cotyledons with acetosyringone showed positive transformation efficiency on GUS analysis. Paz *et al.* (2009) have used half seed methodology successfully; determining final transformation efficiency up to 12%. In maize, immature embryos from different environments had different tissue culture response for maize transformation (Paz *et al*., 2004). In this study, mature and premature half seed explants were used for transformation which revealed that the premature half seed explants had significantly greater regeneration and transformation efficiency than that of mature seeds. The possible reason behind this may be the dormancy and the age of the mature seeds used for the experimental purpose.

The positive role of acetosyringone has been demonstrated on genetic transformation by many plants including recalcitrant species (Godwin *et al*., 1991). Shimoda *et al.* (1990) has been reported that acetosyringone is a phenolic compound that is able to induce the virulence gene in *Agrobacterium* mediated transformation. Addition of acetosyringone to the CCM increased GUS expression in both explants when compared to control (explants co-cultivated without acetosyringone supplementation in CCM).

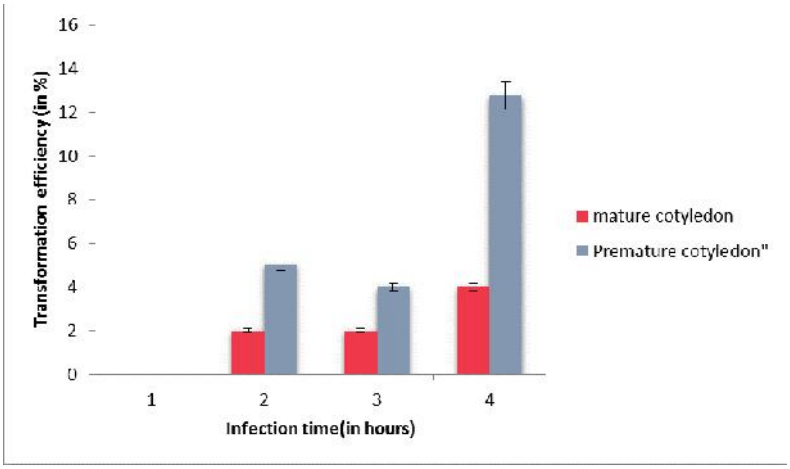

FIGURE 1: Effect of acetosyringone (40 mg/L) and infection time on transformation process

In this present study, significant improvement in GUS expression observed by adding acetosyringone in cocultivation medium while in transformation without using acetosyringone, no GUS expression was observed. This suggests that acetosyringone play an important role in *Agrobacterium* mediated transformation in *Glycine max*

and strongly support the previous report of acetosyringone role during transformation. The T-DNA delivery time depends on *Agrobacterium* strain, vector and explants used. Liu *et al*. (2004) and Ko & Korban (2004) has been reported that 1 hr infection of soybean half seeds with *Agrobacterium* culture was found significant to get high number of transformants and also for survival of explants. While less period (30 min) increased the survival of explants but GUS expression was low. According to many reports, *Agrobacterium* induces necrosis of explants. Similar research was carried out in Soybean by Zia *et al.* (2010) and they reported that the GUS gene expression was highest at infection time of 1h while less time period (30 min) increased the survival of explants but GUS expression was low.

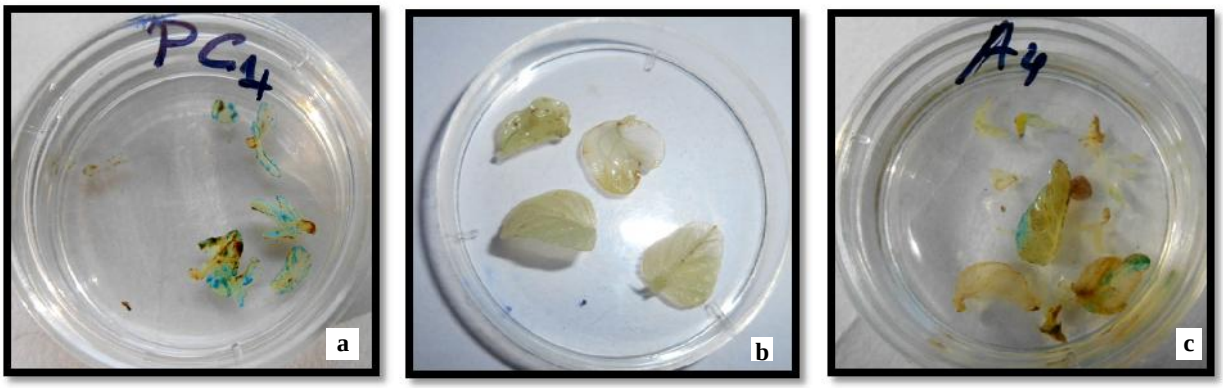

FIGURE 2: Effect of acetosyringone (40 mg/L) and infection time on transformation process Gus expression in leaves of T_0 transformants regenerated from premature **(a)** and mature **(b & C)** half seed explants infected with *A. tumefaciens*: **a)** with AS, infection time 4h; **b)** without AS, infection time 4 h (control); **c)** with AS, infection time 4 h

Effect of dithiothreitol (DTT) on transformation

The results of present study confirmed that GUS gene first increased with increases in the concentration of DTT, and then remains constant at higher concentrations (2 and 2.5mM). Addition of DTT (1-2.5 mM) to CCM containing optimal concentration of acetosyringone (200 µM) and L-cysteine (3.3mM) decreased enzymatic browning (compared to L-cysteine treatment) and increased the percentage of explants showing transient GUS expression. Among the different combinations tested, CCM containing acetosyringone, L-cysteine and DTT (1.5 mM), resulted with a maximum of 13.33 per cent of half-seed explants showing transient GUS expression. This indicates that 1.5 mM of DTT is sufficient for *Agrobacterium* mediated transformation in soybean.

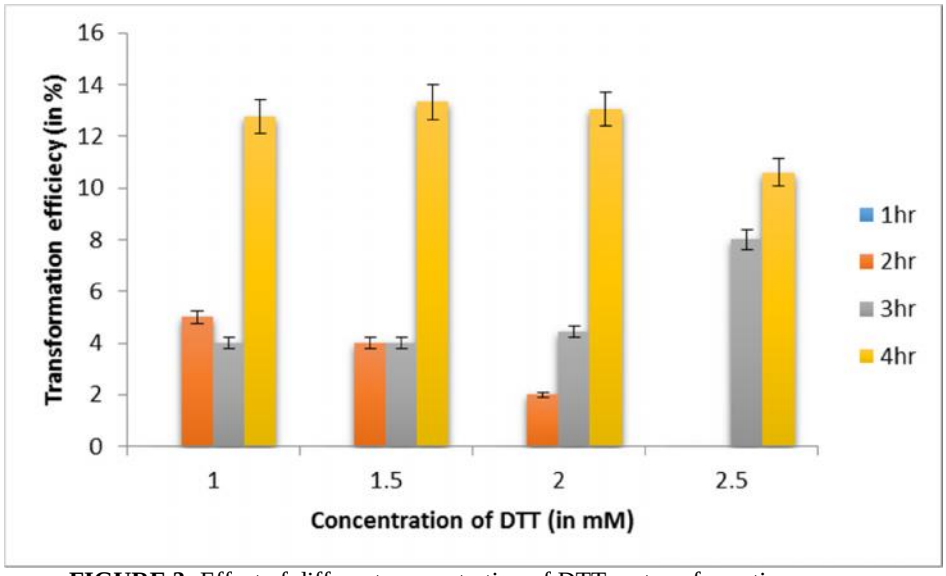

FIGURE 3: Effect of different concentration of DTT on transformation process

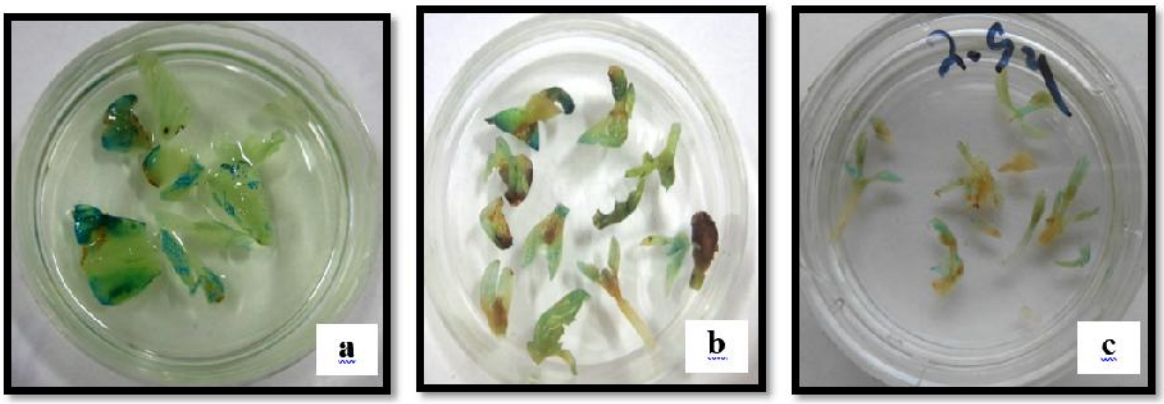

FIGURE 4: Effect of different concentration of DTT on transformation process: Gus expression in leaves of T_0 transformants regenerated from premature half seed explants infected with *A. tumefaciens* LBA4404 harbouring the pCAMBIA1301: **a)** with 1.5 mM DTT, infection time 4 h; **b)** with 2mM DTT, infection time=4h; **c)** with 2.5mM DTT, infection time 4h

Effect of cysteine on transformation

Cysteine is a known inhibitor of PPOs and PODs and enzymatic browning, either directly or indirectly, through the action of its thiol group (Arun, 2009). By reducing wound- and pathogen-defense responses in plants, inhibitors like L-cysteine have the potential to increase the capacity of *Agrobacterium* to infect plant tissues and stably transfer its T-DNA and to increase the frequency of infected cells that remains viable and become transformed (Olhoft and Somers, 2001).

Percentage of explants showing transient GUS expression increased from 12 to 14.89 % for infection time of 4 hrs respectively with the concentration of L-cysteine (4mM) in CCM containing acetosyringone (40mg/l). Further increase of L-cysteine concentration (>4 mM) showed a decline in percentage of explants showing transient GUS expression for half seed explants. L-cysteine, dithiothreitol (DTT), and ascorbic acid are reducing agents that have been successfully used to reduce the effects of oxidizing agents and increase transformation efficiency (Olhoft and Somers 2001) with 2.1 %.

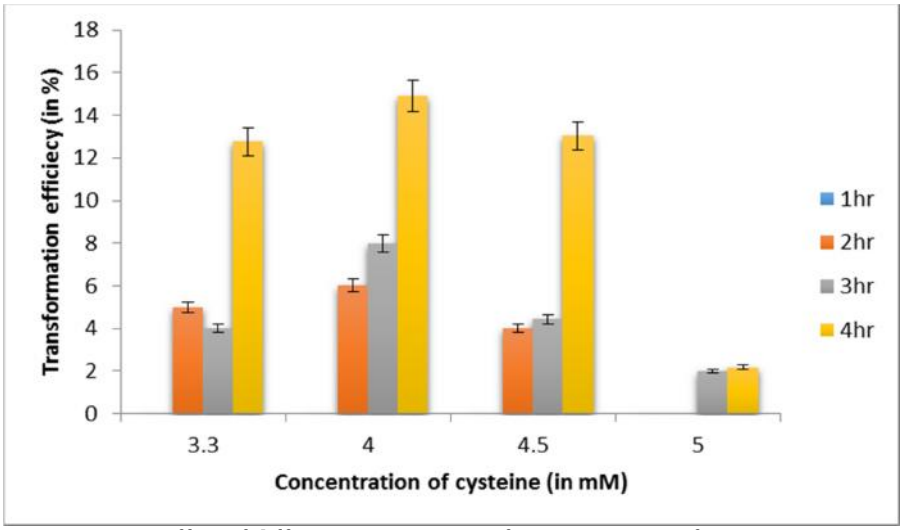

FIGURE 5: Effect of different concentration of Cysteine on transformation process

The addition of cysteine and DTT in co-cultivation media dramatically increased transient GUS activity in all ten cultivars of soybean tested wherein 95% activity was obtained when cysteine was added while 10 per cent GUS expression was obtained without cysteine by Paz *et al.* (2004). It is possible that the antioxidant properties of cysteine and DTT that reduce browning of wounded soybean tissue resulted in better recovery of infected tissues. It is also possible that less oxidized tissue could have improved the interaction between *Agrobacterium* and plant cells as suggested by Olhoft *et al.* (2001). Olhoft *et al.* (2001) described the positive effect of cysteine, DTT and other thiol compounds as protective agent against oxidases in plant tissue. Our observation also suggested that the little higher concentration of DTT and cysteine increases the transformation efficiency while much higher might cause inhibition in the transformation at certain extent.

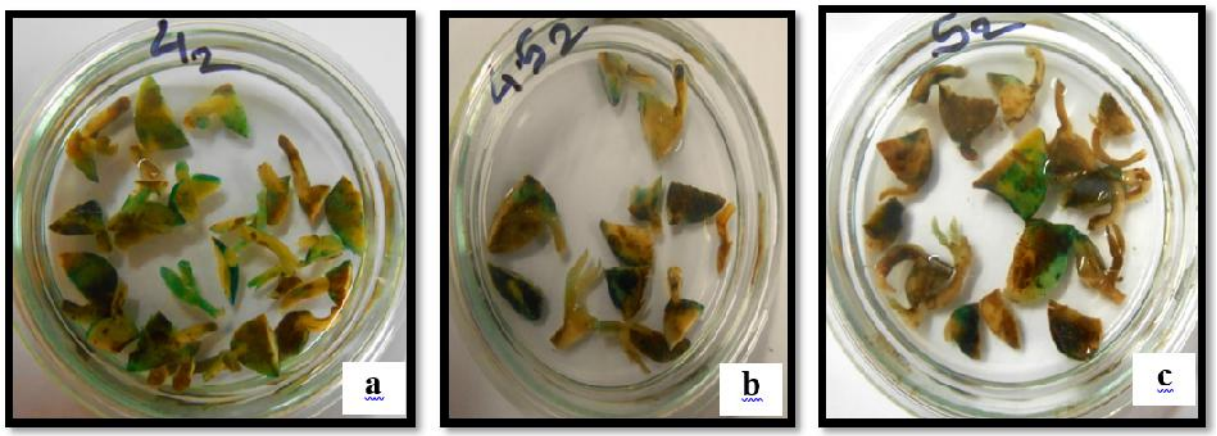

FIGURE 6: Effect of different concentration of cysteine on transformation process. Gus expression in leaves of T_0 transformants regenerated from premature half seed explants infected with *A. tumefaciens* LBA4404 harbouring the pCAMBIA1301: **a)** with 4mM cysteine, infection time 4 h; **b)** with 4.5mM cysteine, infection time 4 h; **c)** with 5mM cysteine, infection time 4 h

Several independent experiments investigating effect of cysteine on *Agrobacterium*-mediated transformation of soybean (JS-9560) was carried out. For this, 3.3, 4, 4.5 and 5mM concentrations of Cysteine used in CCM. It was observed that transient expression of GUS gene first increased with increases in the concentration of cysteine, and then slightly decreased at higher concentrations (4.5 and 5 mM). Olhoft & Sommers (2001) also reported that the addition of cysteine at 3.3 mM to 8.3 mM in the co-cultivation medium increased T-DNA transfer in cotyledonary nodes based on transient GUS expression. Paz *et al.* (2004) also found similar results when 3.3 mM cysteine used in cocultivation medium.

CONCLUSION

It was observed that, the mature cotyledon was having lower transformation efficiency as compare to the premature cotyledon collected just before the maturation. The use of old seeds are greatly affected the efficiency of transformation which may happen due to dormancy and quality of the cotyledons. Presence of acetosyringone in Co-cultivation medium significantly increases the transformation (12.76%) efficiency as compare to lack of acetosyringone in the medium suggesting that acetosyringone plays a cardinal role in infection of plant by *Agrobacterium.* The increase in the infection time significantly improves the transformation efficiency. The slight increase in the concentration of thiol compounds such as DTT and cysteine also significantly improves the transformation process, however higher concentration may adversely affect the transformation efficiency in soybean. The GUS expression analysis revealed that the successful transformation process in soybean using LBA 4404 *Agrobacterium* strain. The presence of blue spots in the leaves and stems showed that GUS reporter gene expressed in whole plants under a control of a constitutive promoter CaMV 35s.

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