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Research Article

SONICATION AND VACUUM INFILTRATION ENHANCED *AGROBACTERIUM RHIZOGENES* MEDIATED TRANSFORMATION IN SOYBEAN

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ABSTRACT

Objective: This study involved the formulation of protocol of *Agrobacterium rhizogenes* - mediated transformation for the detailed study of isoflavones (IFs) metabolism in soybean.

Methods: Cotyledons were separated from 4-day-old soybean seedlings and infected with three different *A. rhizogenes* strains under various time durations of sonication, vacuum infiltration and cocultivated on Murashige and Skoog medium supplemented with various concentrations of acetosyringone. The induced hairy roots were established as a culture with the selection agent hygromycin B. Transgenes integrated in hairy roots were analyzed at molecular level by polymerase chain reaction (PCR) assay.

Results: *A. rhizogenes* strain R1000 harboring pCAMBIA1301 resulted in better transformation efficiency when compared with other strains. The optimum duration of sonication (2 minutes) and vacuum infiltration (2 minutes) enhanced the transformation efficiency up to 76.47%. PCR analyses revealed the integration of transgene in hairy roots lines.

Conclusion: From this study, we could conclude that sonication and vacuum infiltration techniques could be employed to produce genotype independent transgenic soybean hairy root lines and which could be used to study for the improved production of potent anticancer compounds, IFs in soybean.

Keywords: *Agrobacterium*, Isoflavones, Soybean, Sonication, Vacuum infiltration.

INTRODUCTION

Soybean is one of the most considerable important agriculture crops and having a much attention because of its high-value nutritious content and beneficial pharmaceutical compounds. As it belongs to legume family, soybean offers a rich source of vegetable protein to the human diet. In addition to that soybean is also rich in isoflavones (IFs) which are believed to have a vital role in human health. They have shown benefits for the prevention of tumors, prostate cancer, cardiovascular diseases, bone diseases, and other cancers [1]. Among IFs, daidzein, and genistein have been focused for the pharmacological effects. Although various parts of soybean contain different components of IFs, daidzein is present at its highest concentration in root sections and genistein predominantly present in cotyledon [2]. Moreover, daidzein and genistein were identified as major component in soybean roots since they are responsible for inducing the nod genes during nodulation process. Considering the values of daidzein and genistein in beneficial aspect, developing the protocol which utilizes soybean root tissues can effectively pave a way to improve the content of IFs. In this case, hairy roots induced by *Agrobacterium rhizogenes* constitute a valuable and promising source of root-derived phytochemicals [3]. These hairy root cultures exhibit far better growth kinetics in terms of biomass production, fast growth, functional genomics studies, and higher yield of secondary metabolites [4]. Thus, establishing the transgenic soybean hairy root culture system for the investigation of IFs pathway is indispensable.

In soybean, transformation experiments were extensively studied using *Agrobacterium tumefaciens* - mediated transformation and employed various factors such as sonication, thiol compounds, multi needle, microbrush, novel grafting, and vacuum infiltration [5]. In case of *A. rhizogenes*, few works are carried out in soybean, those works included hairy root induction [6], the study of root biology [7], promotors, [8] and seedling transformation [7]. Although *A. rhizogenes* transformation protocol had been standardized in soybean by Owens and Cress [9], there was a large degree of variability observed among soybean genotypes. Hence, it is necessary to establish a standard *A. rhizogenes* - mediated transformation system for Indian soybean cultivars by taking into account of various factors that affect transformation efficiency. Sonication and vacuum infiltration-assisted *A. tumefaciens* - mediated transformation methodology gained much attention due to their success rate in a vast number of plants. Sonication creates micro wounds within the explant tissues and vacuum infiltration infiltrates the *Agrobacterium* into the cavities created by the sonication. Sonication assisted *A. rhizogenes* protocol has been exploited in recalcitrant plants such as *Papaver somniferum* [10] and *Verbascum xanthophoeniceum* [11] and vacuum infiltration methodology has been employed only in *Phtheirospermum japonicum* [12]. But till now, to our knowledge, influence of sonication and vacuum infiltration has not been studied in *A. rhizogenes* - mediated transformation of soybean. Hence, to establish genotype independent *A. rhizogenes* - mediated transformation protocol in Indian soybean cultivars, we have employed both sonication and vacuum infiltration techniques to improve transformation efficiency and transgenic recovery in this study. In case of *A. tumefaciens* - mediated protocol, maximum transformation efficiency of 9.45% is obtained using vacuum infiltration method in soybean [13,14]. Therefore, in this study, we have investigated the optimum conditions required for genotype independent hairy root induction and examined the effect of sonication and vacuum infiltration on transformation efficiency in soybean.

METHODS

Seed germination and cotyledon explant preparation

Three numbers of Indian soybean cultivars (CO-1, CO-2, CO-3) were obtained from Tamil Nadu Agriculture University, Tamil Nadu, India and multiplied in departmental research garden.

Surface sterilization of mature seeds was done by exposing them to chlorine gas for 16 hrs. 4-day-old *in vitro* soybean seedlings were raised by following the method by Theboral *et al.* [15]. The inoculated seeds were then incubated for 3 days under dark at 25±2°C and later transferred into 16/8 hrs light/dark conditions at light intensity of 50 µmol m^{-2} s^{-1} (Cool white fluorescent lamps; Philips, India) for next 1 day. Cotyledons (1.25 cm) were separated from 4-day-old seedlings by gentle twisting against hypocotyl.

***A. rhizogenes* strains and infection**

A. rhizogenes strains A4, R1000 and MTCC 532 (agropine type) were maintained in LB medium (Hi Media, Mumbai, India) supplemented with rifampicin (10 mg/L) [Sigma-Aldrich, India] and solidified with agar (1.75%). A4, R1000 and MTCC 532 strains were wild type of *A. rhizogenes* used in this study. In addition to R1000, strainR1301 harbors R1000 plasmid along with pCAMBIA1301 binary vector which was transformed by freeze thaw method had been used in this study. It contains hygromycin phosphotransferase (*hpt*II) gene as selection marker which confers resistance to hygromycin B and β-glucuronidase (*uidA*) gene as reporter. Both the gene expressions are driven by CaMV35S promoter and *nos* terminator.

Single colony of above strains was inoculated on liquid LB broth (Himedia, Mumbai, India) containing rifampicin (10 mg/L) and incubated in an orbital shaker (Orbitek-Scigenics Biotech Pvt. Ltd., Chennai, India) at 180 rpm for 12-16 hrs at 28°C under total darkness. Kanamycin at 50 mg/L concentration added to LB inoculated with R1301 strain. 12 hrs grown - *A. rhizogenes* cultures (OD_{600nm}:1) were centrifuged (Remi, Mumbai, India) at 8000 rpm for 10 minutes at 28°C and the resulted pellet was resuspended in 35 ml liquid Murashige and Skoog (MS) liquid medium. Acetosyringone at 150 µM concentration (Sigma-Aldrich, India) was added to the *Agrobacterium* - suspensions (approximately 10^{10} cells/ml) 1 hr before infection. Cotyledons of *in vitro* grown 4-day-old seedlings of soybean were injured on abaxial side with hypodermic needle dipped in *A. rhizogenes* suspension.

Effect of sonication and vacuum infiltration treatments on transformation efficiency

For sonication treatment, the wounded cotyledons were transferred into *A. tumefaciens* suspension and sonicated for different time intervals (0, 1, 2, and 3 minutes) at 40 Hz by using water bath sonicator (Model 1510 Branson, Branson Ultrasonics, Japan) and vacuum infiltrated (0, 1, 2, and 3 minutes) at 750 mm of Hg using a desiccator connected to a vacuum pump (Indian high vacuum pumps, Bangalore, India) individually to assess their individual effect on transformation efficiency. For combined effect, after sonication, cotyledons were transferred into fresh *Agrobacterium* suspension and vacuum infiltrated for different time intervals (0, 1, 2 and 3 minutes) at constant pressure of 750 mm of Hg. After treatments, explants were incubated in *Agrobacterium* suspension for 20 minutes with gentle agitation.

Effect of acetosyringone on transformation efficiency

After infection, control (uninoculated) and infected explants were dried in sterile filter paper and transferred to MS basal medium for 4 days of cocultivation in dark. To determine the optimal concentration of acetosyringone for increased infection rate, cotyledons were cocultured on MS medium supplemented with 0, 50, 100, 150, 200 and 250 µM acetosyringone.

After 4 days, all the explants were washed thoroughly with MS liquid medium containing 200 mg/L cefotaxime (Alkem Laboratories, Mumbai, India) and cultured on solid MS basal medium supplemented with 200 mg/L cefotaxime for 2 weeks. The cultures were incubated at 25±2°C under complete darkness. After 10 days of incubation on antibiotic medium, cotyledons were observed for hairy root induction and data were recorded from 10^{th} day of culture to 20^{th} day of culture.

Sensitivity of hairy roots to hygromycin B

To check the sensitivity rate of hairy roots toward hygromycin B, initially hairy roots induced from R1000 infected cotyledons were inoculated on half strength MS liquid medium supplemented with various concentrations (5, 10, 15, 20 and 25/mg/L) of hygromycin B. A culture without hygromycin B was also maintained as control. After 30 days with two subcultures, hairy roots were observed for their surveillance rate and from the observation, minimum inhibitory concentration (MIC) of hygromycin B was determined to select transformed roots.

After standardizing MIC, hairy roots induced from cotyledons which are infected with strain R1301 were subjected to strict selection to eliminate chimera. Briefly, root segments (3 cm) excised from concerned tissues and transferred to MS liquid selection medium which supplemented with 200 mg/L cefotaxime and 15 mg/L hygromycin B to screen putatively transformed roots. Hairy root cultures were subcultured at 10 days interval for three times. The survived hairy roots were used to establish hairy root cultures for further confirmation studies.

Transgene confirmation studies

Assay for β-glucuronidase activity

Gus gene activity and its expression level were assayed by histochemical staining of explants as described by Jefferson [16]. *Gus* assay performed in cotyledon explants infected with *A. rhizogenes* harboring R1301 plasmid and in hairy roots selected using 15 mg/L hygromycin B along with respective controls to check the integration of transgene *gus*.

Molecular confirmation of transgenes in hairy root lines by polymerase chain reaction (PCR) analysis

Hairy roots which were showed constant growth kinetics during proliferation stage were selected for molecular analysis. Five hairy root lines GM1, GM2, GM3, GM4, GM5 and untransformed roots of *in vitro* grown soybean plants were harvested and genomic DNA was extracted using the CTAB procedure. Plasmid DNA was isolated from R1301 strain using alkaline lysis method and used as a positive control at appropriate reactions. The forward and reverse primer combinations used for the amplification of *rol*B, *rol*C, *hpt*II and *gus* genes were 5' ACT ATA GCA AAC CCC TCC TGC 3' and 5' TTC AGG TTT ACT GCA GGC 3' (650 bp), 5' CGA TCC ATG GTC ATT GTT TGC CTC CCT GCT 3' and 5' TTA GCC GAT TGC AAA CTT GCA 3'(550 bp), 5' GAT GTT GGC GAC CTC GTA TT 3' and 5' GTG TCA CGT TGC AAG ACC TG 3' (407 bp), 5' ATG GTA GAT CTG AGG AAC CGA CG 3' and 5' ATG CGT CAC CAC GGT GAT ATC 3' (601 bp), respectively. *rol*B, *rol*C, *hpt*II and *gus* primers were designed using OligoAnalyzer 3.1 tool (integrated DNA Technologies, Coraville, USA [17]. The PCR mixture consisted of 5 ng DNA template, 1.0 µL of primers, 1.0 µL dNTPs, 1.25 µL *Taq* polymerase, and 2.5 µL *Taq* buffer in a total volume of 25 µL. PCR analyses were performed for each hairy root culture with a PTC-100TM thermocycler (MJ Research Inc., San Francisco, USA). Root DNA isolated from the nontransformed plant served as negative control whereas R1301 plasmid was used as a positive control in PCR reactions. After amplification, PCR products were analyzed by electrophoresis on a 1.0% agarose gel (Hi Media, Mumbai, India) using ethidium bromide (Sigma-Aldrich, India) staining and band patterns were documented by UV Gel Documentation system (UVItec, Cambridge, UK).

Genotypic variation in cultivars

After standardizing the various parameters influencing *A. rhizogenes* - mediated transformation using soybean cv. CO-3, cotyledons from other two cultivars were infected with R1301 strain under optimized conditions to test their hairy root induction capacity with reference to their genotypic specificity. Data were recorded after 20 days of infection.

Statistical analysis

Data were analyzed using one-way analysis of variance, and the differences were contrasted using Duncan's multiple range test (DMRT). All the statistical analyses were performed at 5% significant level using SPSS software version 16.0. Graphical representation of analysis was prepared using GraphPad Prism 7 and means±standard error mean were shown for three replicates.

RESULTS AND DISCUSSION

Explant

In this study, we have used cotyledon (Fig. 1b) as explant prepared from 4-day-old *in vitro* raised soybean seedlings (Fig. 1a) to induce hairy roots. After infection, whitish globular callus (Fig. 1c) developed at cotyledonary junction sites within 7 days. Then the differentiation of root primordia (Fig. 1d) from callus was observed followed by the development of matured and branched roots within 20 days. Although various explants available in 4-day-old soybean seedlings, we have used cotyledon as explant based on previous publication [6]. Other studies used cotyledonary junction of seedlings [18] and primary-node [19] as explants.

Conventional use of cotyledon in transformation studies holds several advantages since cotyledon acts as a reservoir for the storage of nutrients and hormones which enhance the cell growth rapidly [19] and large wounding surface area availability for infection [20]. In addition, 4-day-old seedlings provided the mitotic state of target tissues which can affect the transformation efficiency in a positive manner as evidenced by Li *et al.* [7]. Moreover, Mazarei *et al.* [21] obtained vast variation in genotypic response when inoculated *A. rhizogenes* on cotyledonary junction of soybean seedlings whereas Cho *et al.* [6] obtained high and consistent frequency of hairy root induction with cotyledon explants which supported our results obtained in the present study. Lozovaya *et al.* [22] and Weber and Zanettini [23] also used 4-day-old cotyledons for the induction of hairy roots in their studies. In contrast to our results, Owens and Cress [9] obtained better results with stem explants compared to cotyledon explants when infected with R1000 strain.

A. rhizogenes strains

In this study, three different strains were used to evaluate their efficacy in inducing hairy roots. A4 and MTCC 532 were wild type strains, whereas R1301 strain was constructed by transferring pCAMBIA1301 binary vector into R1000 strain for the effective screening of putative transformants. Among the three strains evaluated, R1301 responded well with 35.66% of response with 3.33 umber of root production after 13 days followed by A4 by the percentage of response 28.33 (2.33 number of roots) and MTCC 532 with the percentage of response of 27.66 (2 number roots). Number of days taken for root induction was varying for all three strains. Among the three, R1301 induced roots at the earliest (13 days) followed by A4 (15 days) and MTCC 532 (16 days). The infection frequency of A4 and MTCC 532 was comparatively similar in percentage of response, number of days taken to induce roots and number of roots induced from the cotyledon whereas R1301 showed improved outcome with short DAI (days taken for hairy root emergence after infection) and higher frequency of root induction (Table 1). In preliminary studies, R1000 strain and R1301 constructs were tested to observe for any variation in their infection rate. The obtained results apparently revealed that the infection rate and morphological appearance of roots transformed by R1000 and R1301 were same (data not shown) thus R1301 construct was used for further evaluation studies. This result was supported by Cho *et al.* [6] who observed no difference in morphology of roots raised by K599 and pBI121 harboring K599 construct.

When compared with *A. tumefaciens*, *A. rhizogenes* strains are well suited to produce composite plants due to their virulence, rapid response and simple steps involved. Induction or suppression of auxin (particularly IAA) and cytokinin pathway by *rol* genes may be attributed to the variation observed among transgenic plant tissues as suggested by Nourozi *et al.* [24]. Rapidity of the infection process is determined by days taken for root induction and number of roots produced within that duration. Thus, strain which produces more number of roots within a short duration is more preferable to achieve high transformation efficiency. It is well-known fact that the type of *Agrobacterium* strain, chromosomal background, and virulence capacity of the plasmid influence the transformation efficiency [25].

In general, *A. rhizogenes* strains harboring the agropine type plasmid has the tendency to induce more hairy roots in short duration than other strains. Although all three strains A4, R1301 and MTCC 532 contain agropine type plasmid, the virulence capacity of R1301 is higher than the A4 and hence it showed highest transformation efficiency. Different

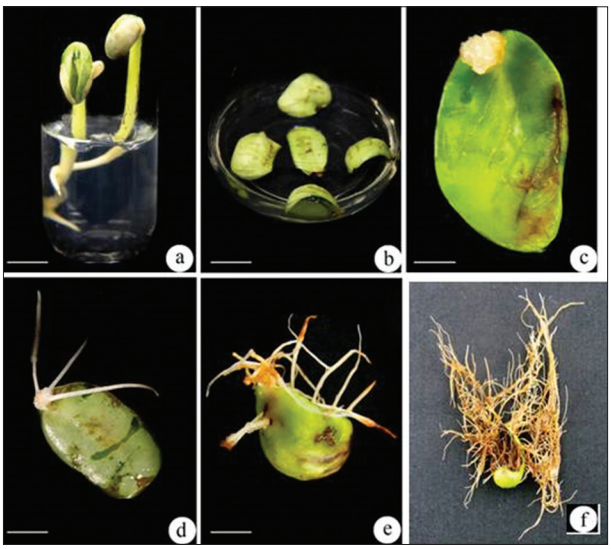

Fig. 1: Induction of hairy roots from cotyledon explants infected with *A. rhizogenes* R1301 strain. (a) 4-day-old soybean seedlings, (b) Cotyledon explants infected and cocultivated on medium containing 150 µM acetosyringone, (c) Callus induction after 7 days of culture, (d) Hairy root induction after 12 days of culture, (e) Hairy root induction from wounded sites of cotyledon treated with sonication and vacuum infiltration for 2 min, (f) hairy root proliferation after 20 days (bar 1 cm)

Table 1: Influence of *A. rhizogenes* strains and acetosyringone on hairy root induction from cotyledons of soybean cv. CO-3

A. rhizogenes strain	Acetosyringone conc. (µM)	Percentage of response	DAI* (days)	Mean number of roots per cotyledon
A4	0	28.33±0.03^{q}	15	2.33±0.03^{l}
	50	31.66±0.03^{o}	14	3.00±0.03^{j}
	100	34.00±0.03^{m}	14	3.66±0.03^{h}
	150	38.66±0.02^{h}	13	4.00±0.02^{ga}
	200	42.33±0.02^{d}	12	5.33±0.0.03^{c}
	250	39.66±0.02^{g}	13	4.33±0.03^{fa}
R1301	0	35.66±0.04^{l}	13	3.33±0.03^{i}
	50	38.33±0.03^{i}	12	4.33±0.03^{f}
	100	43.33±0.03^{c}	11	5.00±0.01^{d}
	150	49.00±0.01^{a}	10	6.33±0.04^{a}
	200	46.66±0.03^{b}	10	5.66±0.03^{b}
	250	40.33±0.03^{f}	11	4.66±0.03^{e}
MTCC 532	0	27.66±0.03^{r}	16	2.00±0.02^{m}
	50	29.33±0.03^{p}	15	2.66±0.03^{k}
	100	32.33±0.03^{n}	15	3.33±0.04^{ia}
	150	36.00±0.02^{k}	14	3.66±0.03^{ha}
	200	40.66±0.03^{e}	13	4.66±0.03^{ea}
	250	37.66±0.03^{j}	14	4.00±0.01^{g}

Data represent the mean±standard error of three experiments and each treatment was repeated thrice. Mean values in a column followed by same letters are not significantly different according to DMRT at 5% level. *Days taken for hairy root emergence after infection

$$\text{Percentage of response} = \frac{\text{Number of cotyledons responded for hairy root induction}}{\text{Total number of cotyledons infected}} \times 100$$

behavior of three strains may be due to the presence of different chromosomal virulence genes and the phenomenon of strain influences on transformation efficiency had already been proved in hairy root cultures of *Solanum xanthocarpum* [26], *Agastache foeniculum* [24], and *Ocimum basilicum* [20].

Even though K599 strain was predominantly used for hairy roots induction in soybean [8], Li *et al.* [7] proved R1000 strain was more efficient than K599 which is in agreement with our results. Similarly, strain MTCC 532 also successfully employed to induce hairy roots in *Solanum xanthocarpum* [26] yet in soybean it could not compete with A4 and R1000 strains. The *A. rhizogenes* R1000 has already been successfully used to induce hairy roots in *Gentiana macrophylla* [25] and *Platycodon grandiflorum* [27] which were corroborated with our results. In addition, we observed some morphological variation in hairy root nature induced by three strains. Hairy roots induced by R1301 were thick and having more emerging branches whereas A4 and MTCC532 strains showed thin roots with more branches. Srivastava *et al.* [20] also observed variation in morphology and growth pattern of hairy roots induced by different strains in *O. basilicum*. Similar to our results, Bansal *et al.* [28] stated that hairy roots induced by R1000 strain were thick whereas A4 strain induced thin roots in *Bacopa monnieri* L. Differential expression of T-DNA genes, variable in copy numbers of T-DNA inserts and positional integration of T-DNA in host genome could be the possible explanation behind the different morphological characters of tissues as suggested by Cho *et al.* [6].

Effect of acetosyringone on transformation efficiency

In this investigation, hairy roots were induced from the cotyledon explants even in the absence of acetosyringone yet it took more days to induce roots. It indicates that acetosyringone is not necessary for hairy root induction from soybean cotyledons, however, addition of acetosyringone in infection and cocultivation medium significantly improved the percentage of response for hairy root induction and mean number of roots per cotyledon meanwhile significantly reduced the days taken for root induction (Table 1 and Fig. 1d). Step-wise increase in acetosyringone concentration gradually improved the percentage of response and number of roots induced at wounded site. 150 micro molar concentration of acetosyringone was proved to be optimal for *A. rhizogenes* R1301 strain and 200 µM acetosyringone was needed to induce *vir* genes expression of A4 and MTCC 532 strains. Under these concentrations, 49.00, 42.33 and 40.66% of cotyledons were responded for hairy root induction and produced 6.33, 5.33, and 4.66 mean number of roots per cotyledon within 10, 12, and 13 days by R1301, A4 and MTCC 532 strains, respectively (Table 1). Further increase beyond optimum concentration notably reduced the percentage of response which subsequently reduced the root number since explants became deteriorated due to bacterial over-growth. Thus, results obtained here apparently confirmed the positive role of acetosyringone in increasing transformation efficiency at optimum concentration.

Acetosyringone is a well-known phenolic compound which improves the *Agrobacterium*-mediated transformation by activating the *vir* genes. Its concentration plays an important role in the transgene delivery and integration. Hence, optimizing the concentration of acetosyringone in infection and cocultivation medium is a prerequisite for obtaining successful transgenic recovery. Obtained results evidently indicated that there was a significant interaction between the type of *A. rhizogenes* and acetosyringone concentration. This notion had already been studied in various plant species including *Glycine max* [8] and *Cannabis sativa* L. [29]. On the whole, in this study, the supplementation of acetosyringone in cocultivation medium enhanced *A. rhizogenes* - mediated transformation efficiency in soybean.

Effect on sonication and vacuum infiltration on transformation efficiency

In this study, among the different time durations (1, 2, 3, and 4 minutes) evaluated, wounded cotyledon explants which were sonicated for 2 minutes showed 64.66% of response for hairy root induction and produced 10.33 mean number of roots per cotyledon. Beyond 2 minutes of sonication, the percentage of response to hairy root induction was reduced which eventually reduced the root numbers (Table 2). Further increase in percentage of response and mean number of hairy roots was obtained with vacuum infiltration and 3 minutes vacuum infiltration was found to be optimum, as under these conditions, 71% of cotyledon explants were responded for hairy root induction with 12.66 mean number of hairy roots per cotyledon. Beyond 3 minutes vacuum infiltration the percentage of response was reduced (Table 2) which may be due to the severe infection.

Further, to increase the percentage of hairy root induction, the sonicated cotyledons were vacuum infiltrated for different time duration in *Agrobacterium* suspension. Among the different time intervals and combinations analyzed, 2 minutes sonication coupled with 2 minutes vacuum infiltration was found to be optimum, as under these conditions, 89.33% of cotyledon explants were responded for hairy root induction with 16.33 mean number of hairy roots per cotyledon (Table 2). On the other hand, the percentage of cotyledons response to hairy root induction and mean number of hairy roots were reduced when explants were treated with either increased sonication time (3 minutes) coupled with 2 minutes vacuum infiltration or increased vacuum infiltration duration (3 minutes) coupled with 2 minutes sonication (Table 2). Sonication and vacuum infiltration treatment not only improved the infection rate, in addition, they encouraged the rapid induction of hairy roots from 7th day onward.

Although *A. rhizogenes* - mediated transformation is well-established in soybean, transformation efficiency still needs to be improved. In general, cotyledonary nodal sites are responsive for *Agrobacterium* infection and efficiently produce roots hence hairy roots were induced only at the cotyledonary nodal sites and no hairy roots were induced from other wounded sites of cotyledon of Indian soybean cultivars. This may due to the recalcitrant nature of Indian cultivars to *Agrobacterium* transformation [14].

In the meanwhile, transformed and nontransformed cotyledons (control) were also having the capacity to induce roots at nodal region after longer time of incubation which was evidenced by the observation of Savka *et al.* [30] in cotyledon explants which may leads to the production of false-positive roots. Thus, as per their suggestion,

Table 2: Influence of sonication and vacuum infiltration treatment on hairy root production in soybean cv. CO-3

Sonication duration (minutes)	Vacuum infiltration duration (minutes)	Percentage of response	DAI (days)	Mean number of roots per cotyledon
0	0	49.00±0.02^{pa}	10	6.33±0.03^{o}
1	-	57.33±0.03^{o}	10	8.66±0.03^{l}
2	-	60.33±0.03^{m}	9	9.00±0.01^{k}
3	-	64.66±0.04^{i}	7	10.33±0.04^{i}
4	-	62.00±0.01^{l}	8	9.66±0.03^{ja}
-	1	63.33±0.03^{j}	10	9.66±0.03^{j}
-	2	68.66±0.03^{ha}	9	10.33±0.03^{ia}
-	3	71.00±0.01^{e}	7	12.66±0.04^{e}
-	4	69.00±0.01^{g}	8	11.66±0.04^{ga}
1	1	68.66±0.03^{h}	7	11.66±0.04^{g}
2	1	72.33±0.03^{d}	7	13.33±0.03^{d}
3	1	70.00±0.01^{f}	10	12.00±0.01^{f}
1	2	80.33±0.03^{b}	7	14.66±0.02^{b}
2	2	89.33±0.03^{a}	7	16.33±0.03^{a}
3	2	76.66±0.03^{c}	9	13.66±0.03^{c}
1	3	62.33±0.03^{k}	9	10.66±0.04^{h}
2	3	58.66±0.03^{n}	9	8.33±0.03^{m}
3	3	49.00±0.01^{p}	10	6.66±0.03^{n}

Data represent the mean±standard error of three experiments and each treatment was repeated thrice. Mean values in a column followed by same letters are not significantly different according to DMRT at 5% level

the induction of transformed roots from the wounded sites other than cotyledonary nodal region is indispensable to obtain putatively transformed roots. To overcome the above said chimera problem, we have tried sonication and vacuum infiltration treatments individually and also in combination during *A. rhizogenes* - mediated transformation studies. These two methods effectively allow the penetration of *Agrobacterium* into plant tissues and interestingly as expected, hairy roots were also induced from wounded sites of cotyledon other than cotyledonary site (Fig. 1e and f) which showed their positive impact on infection rate. Yet, standardization of time periods for sonication and vacuum infiltration is an important prerequest for efficient transformation. Since excess time periods could cause hyper hydration or loss of tissue viability, which means that period should be carefully evaluated [31].

Thus, we have tried 17 different time period and combinations of sonication and vacuum infiltration, among them 2 minutes sonicated and 2 minutes vacuum infiltrated explants produced more roots not only at the cotyledonary nodal sites also in other wounded sites which help us to eliminate chimera roots easily since nontransformed cotyledons are not having the capacity to induce roots other than cotyledonary nodal portion. Longer duration of sonication and vacuum infiltration had inhibitory effect on cells, such as cell lysis, suppression of RNA and protein synthesis of cell walls [31] which confirmed by this study. In such treatments, green colored cotyledon turned into pale color and failed to survive after cocultivation due to severe bacterial growth and cell lysis. Georgiev *et al.* [11] applied 45 seconds sonication to transform the leaf discs of *V. xanthophoeniceum* with *A. rhizogenes* and reported 75% of response for hairy root induction. In a similar fashion, sonication was successfully employed to transform *P. somniferum* [10], In contrast to these results, in this study, cotyledon explants required 2 minutes of sonication for high frequency of hairy root induction and short DAI.

There are several reports describing the positive influence of sonication and vacuum infiltration combination in *A. tumefaciens* - mediated transformation in various plants [14]. However, till date only one report is available in the combination of sonication and vacuum infiltration assisted *A. rhizogenes* mediated transformation [12]. They reported that 10 seconds sonication coupled with 5 minutes vacuum infiltration favored *A. rhizogenes* - mediated transformation of *P. japonicum* whereas we obtained more numbers of hairy roots with 2 minutes treatment of sonication and vacuum infiltration. In soybean, this is the first report on the combination of sonication and vacuum infiltration in *A. rhizogenes* - mediated transformation by which the transformation efficiency was enhanced along with short DAI.

MIC of hygromycin B and hairy root proliferation

Hairy roots obtained from cotyledons infected with R1301 strain under optimum conditions were used to establish hairy root cultures. Initially, to determine MIC of hygromycin B, hairy roots obtained from R1000 infected cotyledons were inoculated in half strength MSliquid medium supplemented with various concentrations of hygromycin B. Among the different concentrations tested, 15 mg/L hygromycin B completely inhibited the growth of induced hairy roots and stopped the emergence of new hairy roots. Lesser concentrations were not effective in inhibiting new root induction, whereas they inhibited subsequent growth of roots. Higher concentration of hygromycin B resulted in complete inhibition of new and existing root growth (Fig. 2).

To establish hairy root lines, hairy roots induced at wounded region of cotyledons which were infected with R1301 strain were selected and their segments were inoculated in MS liquid medium supplemented with 15 mg/L hygromycin B to eliminate chimeric roots. When R1301 transformed roots were inoculated on selection media, growth was disturbed in first subculture, however, they attained their maximum growth rate from second subculture onward since they started to adapt selection pressure with the aid of transgene *hpt*II.

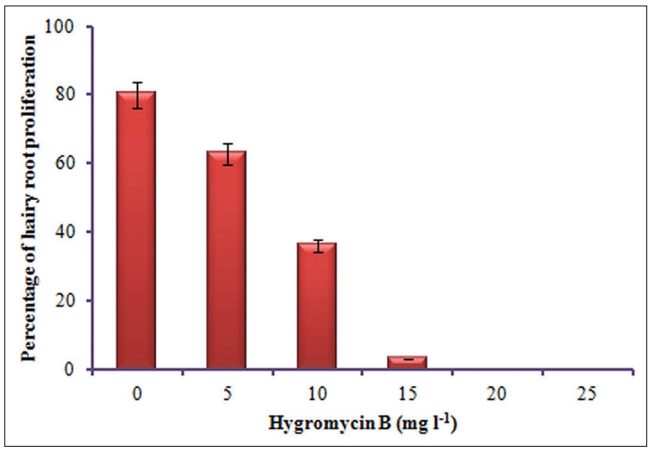

Fig. 2: Sensitivity of soybean cv. CO-3 hairy roots to hygromycin B. Control (0): Treatment without hygromycin B. Hairy root segments were obtained from soybean cotyledon explants infected with *A. rhizogenes* R1000 strain and inoculated on half-strength MS liquid medium and maintained for 40 days including three subcultures. Data were recorded after 40 days of culture. Data are represented as the mean±standard error from three replicates

Hygromycin B is more pronounced selection agent in soybean transformation methods. Since it inhibits polypeptide elongation in protein synthesis process [32], it can strictly inhibit the growth of nontransformed escapes. Extensive *A. tumefaciens* - mediated transformation studies of soybean and other plants used hygromycin B as potent selection agent and the researchers successfully obtained transgenic plants. In case of *A. rhizogenes* - mediated transformation, kanamycin has been broadly used as a selection agent in *G. max* [6]. Of various concentrations tested, 15 mg/L hygromycin B completely inhibited the proliferation of nontransformed roots. In contrast to our results, Guo *et al.* [33] used 20 mg/L hygromycin B for selecting soybean transgenic hairy root lines. However, in this study, hairy roots were unable to grow and proliferated in 20 mg/L hygromycin B. The reason behind this may be due to genotypic difference. Certain studies used both kanamycin and hygromycin B as selection agents to eradicate chimera in *G. max* [7]. In contrast, we have used hygromycin B alone which resulted in complete recovery of transgenic hairy root lines.

Transgenes confirmation studies

Gus expression analysis

Gus histochemical assay was performed to determine *gus* gene expression in transformed hairy root cultures. A blue color was observed in cotyledon (Fig. 3a) and hairy roots (Fig. 3b and c) which were transformed with R1301 strain indicated that *gus* gene was successfully integrated and expressed in hairy roots. Blue color was observed in 75% of hairy roots and an intense blue color was observed in root tips and root nodes of branches. Conversely, roots from wild type *A. rhizogenes* R1000 infected cotyledons failed to show blue coloration up on *Gus* staining (Fig. 3d). Hairy root cultures survived in selection medium produced positive results in *gus* assay. In soybean, complete and intense expression of *gus* in all regions of roots was observed [6,18] whereas Li *et al.* [7] observed escapes among transformed roots infected with strain R1000 harboring binary vector pIG121Hm. Similar to Li *et al.* [7], in this study, we did not get the complete expression in *Gus* analysis, which might be due to genotype difference since CaMV35S promoter driven expression of *gus* can reach variable level depends on expression [8]. Various promoters were fused with *gus* and analyzed for their expression, in which different *gus* expression patterns were obtained in soybean transgenic hairy roots [8].

Molecular confirmation of transgenes by PCR analysis

Hairy roots survived in 15 mg/L hygromycin B supplemented medium were used for further molecular studies. After 30 days in selection

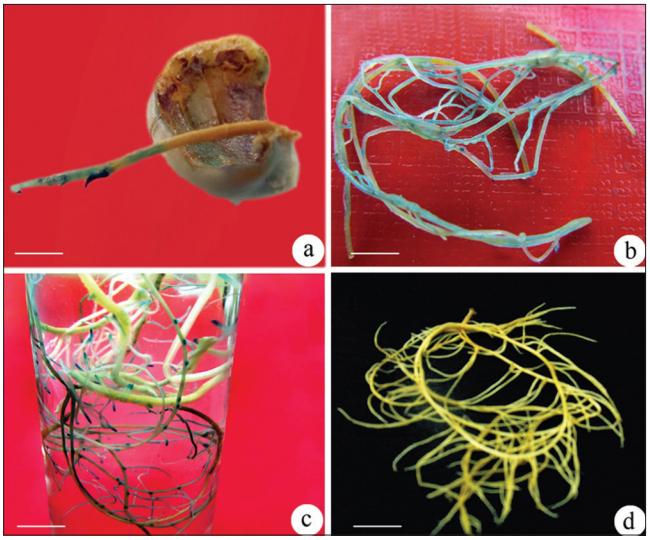

Fig. 3: *Gus* expression in cotyledon and hairy roots induced from A. rhizogenes R1301 infected 4-day-old cotyledons of soybean cv. CO-3. (a) Cotyledon showing *Gus* expression after 7 days of infection, (b and c) expression of *Gus* in proliferated hairy roots, (d) hairy roots induced by *A. rhizogenes* R1000 failed to express *Gus* (bar 1 cm)

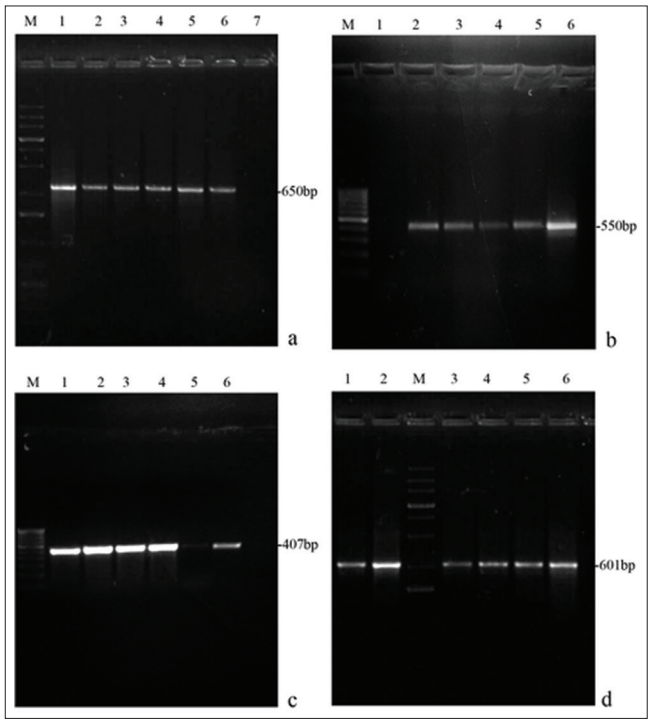

Fig. 4: Polymerase chain reaction (PCR) confirmation of transgenes in putative hairy root lines induced from cotyledons infected with *A. rhizogenes* R1301 strain. (a) PCR analysis of *rolB* gene with 650 bp size product lane M: 100 bp marker, lane 1: Plasmid R1301, lane 2-6: DNA samples from transformed hairy root lines, lane 7: DNA sample from nontransformed root as negative control, (b) PCR analysis of *rolC* gene with 550 bp size product lane M: 100 bp marker, lane 1: DNA sample from nontransformed root, lane 2: plasmid DNA of R1301, lane 3-6: DNA samples from transformed hairy root lines, (c) PCR analysis of hygromycin phosphotransferase gene with 407 bp size product lane M: 100 bp marker, lane 1: plasmid DNA of R1301, lane 2-4 and 6: DNA samples from transformed hairy root lines, lane 5: DNA sample from nontransformed root, (d) PCR analysis of *gus* gene with 601 bp size product lane 1 and 3-6: DNA samples from transformed hairy root lines, lane 2: Plasmid DNA of R1301, lane M: 100 bp marker, lane 7: DNA sample from nontransformed root

media, genomic DNA was isolated from hairy roots were analyzed by PCR with *rol*B, *rol*C, *gus,* and *hpt*II gene specific primers. The results showed that hairy roots survived in selection media produced positive band patterns for *rol*B (650 bp) (Fig. 4a), r*ol*C (550 bp) (Fig. 4b), *hpt*II (407 bp) (Fig. 4c), and *gus* (601 bp) (Fig. 4d) genes which confirmed complete transformation of hairy roots. All hairy root cultures (GM1-GM5) produced the expected size of all four transgenes. Control lines, untransformed soybean roots failed to amplify *rolB* and *rolC* genes (Fig. 4a and b) whereas R1000 infected hairy root culture failed to amplify *gus* and *hpt*II genes (Fig. 4c and d). Molecular confirmation of transgenes is a critical step in hairy root lines since during *Agrobacterium* infection nontransformed roots may arose from the explants [30]. Thus, in this study, we have confirmed the presence of transgenes through PCR analysis. All hairy root lines produced expected size bands on PCR amplification for *rolB, rolC, hpt*II, and *gus* genes which were due to strict selection methodology followed in this study.

Transformation efficiency

When cotyledons of soybean cv. CO-3 infected with R1301 under optimized conditions, the total transformation efficiency of 76.47% was obtained in this study (Table 3). Transformation efficiency was calculated from *gus* expression and PCR analysis of transformed hairy roots. Hygromycin B resistant hairy roots showed positive results in *gus* and PCR analysis. Cho *et al.* [6] also obtained 100% of *gus* and GFP expression in kanamycin resistant hairy roots of soybean which is corroborate with our results. Maximum 95% of transformation efficiency was obtained by Cho *et al.* [6] using K599 strain based on *Gus* expression in soybean hairy root induction. While Mazarei *et al.* [21] and Weber and Zanettinni [23] obtained 50% and 52% of transformation efficiency in cotyledon explants infected with K599 strain. Li *et al.* [7] also employed R1000 strain to induce hairy roots yet they obtained 25.4% of transformation efficiency in soybean which was in contrast to our results. We have obtained 76.47% of transformation efficiency with same strain by employing sonication and vacuum infiltration treatments which might improved the transformation efficiency of Indian soybean cultivar CO-3 in this study.

Effect of genotypes on transformation efficiency

Variation in response to each cultivar depends on number of factors including explant, bacterial strains, etc. Thus, selection of cultivar for the investigation plays a decisive aspect in determining the success of the study. All three cultivars responded to *A. rhizogenes* infection and produced hairy roots from explants. The explants produced callus within 7-10 days and the callus started to produce roots within 25 days. Among ten cultivars, there were minor fluctuations observed in their response and root induction efficiency. Of all, CO-3 showed higher percentage of response (85.99 %) and induced a higher number of roots (14.6) and CO-1 showed least response (66.66) for root induction and taken more days (13) to induce roots (Table 4). In all aspects (high percentage of response, DAI and higher number of root induction), cultivar CO-3 out rightly showed better performance and hence preferred for further studies.

Hairy root formation is not only dependent on *A. rhizogenes* strains, but also dependent on the host plant genotype [30]. Each soybean variety responded differently for the present evaluation and this characteristic has already been discussed by various investigators [6,18,30]. Based on their investigations, a genotype which is having short DAI, a large number of hairy roots and high transformation frequency can be described as desirable genotype for *A. rhizogenes* transformations. However, Cao *et al.* [18] concluded that no single genotype possessed all these characters which are in agreement with our results. Although,

Table 3: Transformation efficiency of hairy root induction from cotyledon explants of soybean cv. CO-3 infected with *A. rhizogenes* R1301 strain

Total number of infected cotyledons	Percentage of response	DAI (days)	No. of hairy roots induced per explant	No. of hygromycin B resistant roots	No. of *Gus* positive roots	No. of PCR positive roots	Transformation efficiency (%)
50	90.0	7	17	13	13	13	76.47

PCR: Polymerase chain reaction. All the explants were sonicated and vacuum infiltrated for 2 min with *A. rhizhogenes* strain R1301 and cocultivated in the presence of acetosyringone (150 µM). Data were obtained after 50 days of culture. Hairy roots induced from an explant were inoculated individually in selection medium and based on the results obtained after PCR analysis, the transformation efficiency was calculated:

$$\text{Transformation efficiency} = \frac{\text{Number of PCR positive hairy roots}}{\text{Number of hairy roots induced per explant}}$$

Table 4: Influence of genotypes on hairy root induction from cotyledons of Indian soybean cultivars infected with *A. rhizogenes* strain R1301

Cultivar	DAI (days)	Percentage of response	Mean number of roots induced per explant
CO-1	11	66.66±0.21^{c}	8.3±0.15^{c}
CO-2	10	72.45±0.13^{b}	10.4±0.21^{b}
CO-3	7	85.99±0.15^{a}	14.6±0.13^{a}

Explants from three cultivars were sonicated and vacuum infiltrated for 2 min with *A. rhizogenes* strain R1301 and cocultivated in the presence of acetosyringone (150 µM). Data were obtained after 30 days of culture. Data represent the mean±standard error of three experiments and each treatment was repeated thrice. Mean values in a column followed by same letters are not significantly different according to DMRT at 5% level

three varieties showed better performance, each exhibited its better characteristics in each category of the desirable characters, none of the cultivar showed better performance in all three categories. Conclusively, CO-3 variety which had a short DAI and higher number of root induction rate has been selected for further studies. Cao *et al.* [18] suggested that short DAI were important because of space and time saving.

Although minor differences were observed in the percentage of response, mean number of roots, and time taken for root induction, the developed protocol was applicable to transform all the cultivars tested in the present work. In general, *A. rhizogenes*-mediated transformation may not be genotype-dependent if cotyledons were used as explants [6,23].

Contrary, Owens and Cress [9], Savka *et al.* [30] and Mazarei *et al.* [21] observed major differences among varieties in response to *A. rhizogenes* infection and diversity in transformation efficiency which ranged from 5% to 90% whereas in our study and Cho *et al.* [6] observed minor differences in response among genotypes. Optimized protocol and cotyledon explant used in this study could be the possible reason for the improved transformation efficiency of the cultivars as suggested by Cho *et al.* [6]. Variance observed in morphological characters and response among soybean genotypes was a common phenomenon as observed by Cho *et al.* [6] but disagreeable with results of Savka *et al.* [30] who observed no difference in morphological appearance of hairy root cultures of various soybean genotypes.

CONCLUSION

From this study, we may conclude that sonication and vacuum infiltration techniques could be employed to produce genotype independent transgenic soybean hairy root lines from 4-day-old cotyledons of Indian soybean cultivars when transformed with *A. rhizogenes* strain R1301 and cocultivated with the supplementation of 150 µM acetosyringone. This methodology could be used for the improved production of potent anticancer compound, IFs in soybean.

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