Somatic embryogenesis and Agrobacterium mediated genetic transformation in Indian accessions of lucerne (Medicago sativa L.)

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Plant regeneration in lucerne (Medicago sativa) is highly genotype dependent and inspite of considerable information on in vitro regeneration in this species the Indian genotypes have not been investigated so far. A system of recurrent somatic embryogenesis (RSE) was established for the first time in Indian accessions of lucerne and utilized for Agrobacterium mediated genetic transformation. Seeds, hypocotyls and cotyledons of LLC-3, C-10, A-3 and IL-75 accessions and ovary explants of 5 somaclones of LLC-3 were used and globular shaped somatic embryos were observed in all of the cultures. All the developmental stages of somatic embryogenesis were observed in ovary culture of one somaclone only. These somatic embryos have been undergoing cycles of RSE and the system has perpetuated for 24 months. For Agrobacterium mediated genetic transformation, this system of RSE required creation of injury in individual somatic embryos for enhanced transformation efficiency that limited its utilization for large-scale transformation experiments. An efficient system to overcome the requirement of creation of injury was developed in which pre-culture of somatic embryos on 2,4-D supplemented medium prior to their co-cultivation with Agrobacterium and supplementation of 2,4-D in co-cultivation medium could significantly improve the transformation efficiency.

Keywords: Medicago sativa, lucerne, somatic embryogenesis, recurrent somatic embryogenesis, plant regeneration, genetic transformation

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Introduction

Alfalfa (Medicago sativa L.), commonly known as lucerne, is the most important forage crop in the world. It is cultivated over an area of 35 million hectares1. In India, it is the third important forage crop after sorghum and berseem and occupies one million hectare area providing 60 to 130 tonnes of green forage per hectare2. Lucerne has got some inherent problems for its genetic improvement such as uncontrolled pollination, inbreeding depression and the non-availability of desirable gene pool in the germplasm. Because of these hurdles the breeding activities in the last century have not resulted in any significant improvement in its yield potential3. Genetic transformation of lucerne with genes for sulphur containing amino acids, tolerance to various biotic and abiotic stresses would improve its nutritive value and yield. Hence, optimization of in vitro regeneration and genetic transformation protocols is the most essential component for application of biotechnology for genetic improvement of this crop. Tissue culture and genetic transformation systems for lucerne have been developed in a number of studies4-8. Genotype and explant have been identified as important factors that influence in vitro regenerative potential of lucerne8,10. Genotypic influence on plant regeneration in lucerne is so pronounced that in various countries regenerative genotypes have been identified and the genetic transformation work is limited to these regenerable genotypes only. Since this crop is highly cross-pollinated and heterozygous and heterogeneous in its genetic architecture, a single accession (variety) has many genotypes in it and identification of regenerative genotypes is compounded by two factors (i) identification of regenerative accessions, and (ii) regenerative genotypes in them. Once a regenerative genotype is identified, it is maintained through vegetative propagation (stem cutting), micropropagation, RSE or artificial seeds as a source of regenerative material for further biotechnological work.
However, the Indian accessions and genotypes of lucerne have not been tested so far for plant regeneration. The transient GUS expression in the co-cultivated calli of cotyledon and hypocotyl explants has been reported in only one study. In the present investigation, attempt was made to identify regenerative accessions and somaclones (single genotype) of lucerne. Hypocotyl, cotyledon and seed explants of four Indian accessions and ovary explants of five somaclones of LLC-3. Flower buds, harvested just before anthesis, and seeds were surface sterilized. The cotyledon and hypocotyl segments were excised from one-week-old aseptically grown seedlings. Ovaries were isolated from sterilized floral buds.

**Materials and Methods**

Experimental material used in the present study comprised seeds, hypocotyls and cotyledons of LLC-3, C-10, A-3 and IL-75 accessions and ovary explants of 5 somaclones of LLC-3. Flower buds, harvested just before anthesis, and seeds were surface sterilized. The cotyledon and hypocotyl segments were excised from one-week-old aseptically grown seedlings. Ovaries were isolated from sterilized floral buds.

**Culture Initiation and Somatic Embryogenesis**

Calli were induced from seeds, hypocotyls and cotyledons on 2 mg/L 2,4-D, 1 mg/L NAA and 0.2 mg/L BAP supplemented MS medium. The callus induced from these explants was subcultured after three weeks on the same callus induction medium for another three weeks and then transferred to 10 mg/L 2,4-D and 1 mg/L kinetin supplemented SH medium for four days. Subsequently, this callus was subcultured on 150 μM proline supplemented SH medium (SHP medium) for somatic embryogenesis. Callus from ovaries was induced on 5 mg/L 2,4-D and 1 mg/L kinetin supplemented MS medium and subcultured after 12 days to basal MS medium for somatic embryogenesis. Callus induction frequency was recorded as percentage of total number of explants producing callus. Callus colour was recorded on a visual scale from 1 to 4 (1: watery, 2: granular, 3: friable). Callus growth was recorded on a visual scale from 1 (very poor callus growth) to 4 (profuse callus growth).

**Agrobacterium Mediated Genetic Transformation**

Agrobacterium tumefaciens strain EHA-105 harbouring binary vector pCambia-1301 was used for transformation studies. Plasmid pCambia-1301 carries hygromycin resistance gene under the control of CaMV-35S promoter as selectable marker and gus gene driven by CaMV-35S promoter as a scorable marker. GUS gene was interrupted by catalase intron to prevent its expression in Agrobacterium (Fig. 1). Agrobacterium was grown overnight in Yeast Extract Mannitol (YEM) medium supplemented with kanamycin (50 mg/L) and rifampacin (25 mg/L) at 28±2°C. In the pre-induction method, 100 μM acetosyringone was also added in YEM medium for growing Agrobacterium. The bacterial pellet was obtained by centrifugation (4000 rpm, 15 min at room temperature) and resuspended in MS media (O.D. of 1) with or without 100 μM acetosyringone. Somatic embryos, swollen somatic embryos (callus) and leaves were used as explants for co-cultivation. In one experiment, somatic embryos were cultured on 2, 4-D (5 mg/L) and kinetin (1 mg/L) supplemented MS medium (MSDK) for six days prior to their co-

![Fig. 1 — Plasmid map of pCambia 1301 used for Agrobacterium mediated transformation of Lucerne.](image-url)
cultivation with *Agrobacterium*. The explants (with or without injury) were immersed in the bacterial suspension and put under vacuum for 10 min to infiltrate *Agrobacterium* into the explants. Explants were blot dried and transferred to MS plates with or without acetosyringone (100 μM). MSDK pre-cultured somatic embryos were also cultured on the MSDK medium. Co-cultivation was continued at 28±2°C for three days in dark. The explants were rinsed thoroughly with 250 mg/L cefotaxime supplemented MS medium to kill the *Agrobacterium*, blot dried and transferred to cefotaxime (250 mg/L) and hygromycin (25 mg/L) supplemented MS medium selection plates (MSCH) for two months following fortnightly subculture. Surviving somatic embryos undergoing RSE were transferred to MSCH-1 (250 mg/L cefotaxime and 37.5 mg/L hygromycin) for one month and then to MSCH-2 (250 mg/L cefotaxime and 50 mg/L hygromycin) selection plates. Selection plates were kept under 16-8 h light-dark photoperiod at 26±2°C.

GUS expression was histochemically assayed with 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) as substrate. GUS assay staining solution was prepared with 10 mM EDTA, 50 mM phosphate buffer (pH 7.0), 0.1% Triton X-100, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 20% methanol, 1 mM X-Gluc. Transient GUS expression was scored just after co-cultivation. Explants for stable GUS expression were taken from selection plates.

**Statistical Analyses**

The experimental design used for callus induction frequency of seed, hypocotyls and cotyledon was 2 factorial completely randomized design with 10 replications. There were four levels of first factor (accession) and three levels of second factor (explant). Callus induction frequency of ovary explant was analysed in completely randomized design. Five to seven explants were cultured in one flask and mean response of one flask constituted one replication. The visual scores given for callus growth, colour and texture were analysed using a non-parametric Kruskal-Wallis statistics since these data were recorded on an ordinal scale.

An explant showing one or more GUS blue spots was recorded as positive. Transformation frequency was calculated as the number of GUS expressing explants divided by the total number of explants assayed and expressed as per cent. All the experiments were carried out in a completely randomized design with 5 to 7 replications. Average GUS expression frequency from 10 to 15 explants constituted one replication.

**Results and Discussion**

**Callus Induction, Growth and Quality**

Cultured seeds, cotyledon and hypocotyl explants exhibited callus induction within three to five days after inoculation. Analysis of variance for callus induction frequency showed significant differences for the main effects of accession and explants. The interaction effect of these factors was non-significant (Table 1). The maximum callus induction frequency in A3 (93.33%) was statistically similar to that of C10 (90.17%). Minimum callus induction frequency recorded in IL-75 was found to be statistically at par with the callus induction frequency of LLC-3 and C-10 (Table 2). Among the three explants, hypocotyls (91.88%) and seed (89.75%) were the best explants for the callus induction frequency whereas the cotyledon explants exhibited comparatively poor response (81.88%). The growth of callus varied significantly in different accessions but explant differences for this trait were non-significant. The callus of accession A-3 was found to be the fastest growing while the slowest growing callus was observed in accession IL-75 (Fig. 2). While accession

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>S.S.</th>
<th>M.S.S.</th>
<th>Fcal</th>
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<td>2</td>
<td>2220.438</td>
<td>1110.219</td>
<td>5.32**</td>
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<td>Explant</td>
<td>3</td>
<td>1995.000</td>
<td>6665.000</td>
<td>3.19*</td>
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Significant at 5% level of significance,
**Significant at 1% level of significance.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Seed</th>
<th>Hypocotyl</th>
<th>Cotyledon</th>
<th>Mean</th>
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<tbody>
<tr>
<td>LLC-3</td>
<td>87.00</td>
<td>89.00</td>
<td>76.50</td>
<td>84.17 [b]</td>
</tr>
<tr>
<td>C-10</td>
<td>91.00</td>
<td>92.50</td>
<td>87.00</td>
<td>90.17 [ab]</td>
</tr>
<tr>
<td>A-3</td>
<td>93.00</td>
<td>92.00</td>
<td>95.00</td>
<td>93.33 [a]</td>
</tr>
<tr>
<td>IL-75</td>
<td>88.00</td>
<td>94.00</td>
<td>69.00</td>
<td>83.67 [b]</td>
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<tr>
<td>Mean</td>
<td>89.75 (ab)</td>
<td>91.88 (a)</td>
<td>81.88 (c)</td>
<td>87.83</td>
</tr>
</tbody>
</table>

Mean callus induction frequencies in a row ( ) and column [ ] with different alphabets are significantly different at 5% level of significance.
differences for callus colour were significant, explant differences for this trait were non-significant. Maximum and minimum callus colour responses were observed in LLC-3 and IL-75, respectively (Fig. 2). The visual callus colour score of 3.55 observed in LLC-3 indicated that most of the callus of this accession was green. Callus texture was found to vary significantly in different explants and accessions. The maximum and minimum callus texture responses were observed in IL-75 and C-10, respectively. The accession differences for callus texture were not as pronounced as the explant differences and the maximum callus texture response was observed in cotyledonary calli (Fig. 2).

Callus was initiated very quickly (3 to 4 d of inoculation) from ovaries. Mean callus induction frequency (Fig. 3) in different somaclones varied from 33.33% (LLC-3-3) to 63.66% (LLC-3-2). Poor callus growth, callus colour and callus texture was recorded in somaclones LLC-3-3 and LLC-3-4 (Fig. 4). Other somaclones were comparable to these quality parameters.

Callus induction in *M. sativa* had been reported to be controlled by one locus with complete dominance and random chromatid segregation\(^15\). In this model, genotypes CCCC, CCCc, CCcc, Cccc would produce callus and genotype cccc would not produce callus. Depending on the frequency of alleles C and c, the relative proportion of these four genotypes would vary in different accessions and result in differential callus induction response as observed in the present investigation. Significant genotypic differences for callus growth as observed in the present investigation has also been reported earlier by various workers\(^16\)-\(^18\). In one of the earlier studies a wide range from 62 mg for cv. Chilean hypocotyl explants to 720 mg for cv Angus cotyledons has been reported\(^19\). *In vitro* callus response from the ovary explants of five somaclones of LLC-3 was significantly different. This variation may be attributed to somaclonal variation that has also been recorded at morphological, biochemical (isozyme) and molecular (RAPD) levels in these somaclones in our laboratory (unpublished data).

**Somatic Embryogenesis and Plant Regeneration**

The callus surface was consistently smooth on callus induction and as well as auxin shock medium. Globular shaped somatic embryos started to initiate after 12 to 15 d of transfer of callus to somatic embryo induction medium (SHP or MS) and the cultures were full of such somatic embryos after 25 to 30 d of culture. Range of somatic embryogenesis in the calli of four accessions (Fig. 2) was rather narrow (92-94.9%). Cotyledonary calli, which had scored maximum callus texture response also exhibited maximum somatic embryogenesis response (100%).
Mean globular shaped somatic embryo induction response of different somaclones was comparable and varied from 90.3% (LLC-3-3) to 95.3% (LLC-3-2). Somatic embryos of all of the accessions and somaclones (except LLC-3-2) remained arrested at globular stage without any further development. In the somaclone LLC-3-2, somatic embryos exhibited all the stages of somatic embryogenesis, such as globular heart, torpedo and cotyledonary embryos (Fig. 5). However, the rate of conversion of these embryos into plantlets was quite poor (0.2%), but these embryos exhibited tremendous potential for RSE. On further transfer of these embryos to plain MS medium globular shaped somatic embryos initiated from whole of the surface (Fig. 5). These somatic embryos have been repeating the same RSE cycle since last 24 months. Plants regenerated through this system have been vegetatively propagated and found to have the regenerative potential through somatic embryogenesis.

In contrast to earlier reports about the significant genotypic differences for somatic embryogenesis, different accessions and somaclones in the present investigation were comparable for globular shaped somatic embryo induction. Genotypic differences became apparent for maturation of somatic embryos as the somatic embryos of only one somaclone exhibited all the stages of somatic embryogenesis. The callus quality (colour, texture) of this somaclone was comparable to that of some of the other somaclones and accessions. These results could be explained by considering the inheritance of callus production and plant regeneration suggesting that these two traits are controlled by different genetic factors. RSE in lucerne was for the first time described in 1983 and later reported by many other workers. In the earlier reports, cultures undergoing recurrent somatic embryogenesis could also be maintained for two years. We have successfully maintained the recurrent somatic embryogenic potential for twenty-four months and the cultures are still undergoing RSE.

*Agrobacterium Mediated Genetic Transformation*

The tremendous potential of somatic embryos to undergo RSE prompted us to utilize them for *Agrobacterium* mediated genetic transformation as transformation of even single somatic embryo or its part would lead to development of large number of genetically transformed somatic embryos. RSE has also been reported as a useful system for transformation of walnut. However, this system has not been widely utilized in lucerne because of poor response of somatic embryos to *Agrobacterium*.

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**Fig. 5** — RSE and GUS expression in lucerne: (a-d), Developmental stages of somatic embryogenesis: (a), globular; (b), heart; (c), torpedo; and (d), cotyledonary; (e), RSE in globular and heart shaped embryos developed on radicle of germinated embryo; (f), GUS expression in leaf segment; and (g), in somatic embryo.
In the present investigation, we have been successful in improving *Agrobacterium* mediated transformation efficiency of the somatic embryos. Somatic embryos, swollen somatic embryos (callus) that were about to exhibit RSE and leaf segments from *in vitro* germinated seeds were used for *Agrobacterium* mediated genetic transformation. Leaves were included in the experiment as a positive control since this explant has been shown to exhibit very high transformation efficiency. Explant differences for *Agrobacterium* mediated genetic transformation were significant. Maximum frequency of GUS expression (Table 3) was scored in leaves followed by somatic embryos (Fig. 5). No GUS expression was observed in callus. Our results corroborated the earlier observations of one of the studies on poor transformation efficiency of somatic embryos. To improve the transformation efficiency of somatic embryos, two methods were used for effective activation of *vir* region of *Agrobacterium*. Since acetosyringone has been reported to be a very effective inducer of *vir* region during pre-induction method, 200 μM acetosyringone was supplemented in YEM medium used for initiation of *Agrobacterium* cultures as well as in co-cultivation medium. In the second method, injury was created in the explants before their co-cultivation with *Agrobacterium*. Although the main effects of two methods on genetic transformation were not significant, these methods exhibited significant interaction with the explant which was evident from the observation that the magnitude of increase in transformation efficiency on injury of somatic embryos was significantly more than that of leaf explant. However, transformation of calli was not successful by any of these methods. Findings of the present investigation suggest that acetosyringone might not be as good a *vir* region inducer in lucerne as the natural exudates (phenolics) released on injuring the explant. In one of the earlier studies also it has been reported that inclusion of acetosyringone in co-cultivation medium does not improve the transformation efficiency of lucerne. Injuring somatic embryos of one to two mm size is a laborious task which limits large-scale *Agrobacterium* mediated transformation of somatic embryos. Injury of explants in dicots incites wound response in the form of secretion of *vir* region inducing phenolics and multiple cell divisions at the site of injury. Injury of explants also increases the surface area for attachment of *Agrobacterium* to plant cells. Preculture of explants on a high 2,4-D medium prior to co-cultivation has been shown to improve *Agrobacterium* mediated genetic transformation efficiency. Enhancement of GUS activity on raising the 2, 4-D concentration in the co-cultivation medium from 0 to 6 mg/L has earlier been reported. Preculturing the explants on 2, 4-D supplemented medium or its inclusion in co-cultivation medium enhances the rate of cell division and, thereby, makes the tissue more competent for *Agrobacterium* infection. Our results also corroborate these observations. A significant improvement of 25.7% over control was observed in transformation efficiency when somatic embryos, pre-cultured on 2, 4-D medium, were used as explants for transformation (Table 4). Improvement in transformation efficiency could further be enhanced when 2, 4-D pre-cultured somatic embryos were co-cultivated with *Agrobacterium* on 2, 4-D supplemented medium. These results suggested that with the use of 2,4-D, injuring embryos may be avoided and they could be directly used for large-scale transformation. During our study, *Agrobacterium* suspension was infiltrated into the explants by applying vacuum. This method is essentially used during *in planta* transformation of *Arabidopsis*. In our initial experiments, we had also recorded promotive effect of vacuum infiltration of *Agrobacterium* suspension into the explants on transient GUS expression frequency.

<table>
<thead>
<tr>
<th>Table 3 — Effect of co-cultivation methods on transformation efficiency of somatic embryos, leaves and calli</th>
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<tr>
<td><strong>Explant</strong></td>
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<tr>
<td>Somatic embryo</td>
</tr>
<tr>
<td>Leaf</td>
</tr>
<tr>
<td>Calli</td>
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<td><strong>Transformation efficiencies in row ( ), column [ ] and the body of table with different alphabets are significantly different at 5% level of significance.</strong></td>
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<th>Table 4 — Effect of 2,4-D pre-culture on transformation efficiency of somatic embryos</th>
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<tbody>
<tr>
<td><strong>Treatment</strong></td>
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<tr>
<td>Somatic embryos (No 2,4-D pre-culture)</td>
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<tr>
<td>Somatic embryos pre-cultured on 2,4-D</td>
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<tr>
<td>Somatic embryos pre-cultured on 2,4-D + 2,4-D in co-cultivation medium</td>
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<td>Transformation efficiencies with different alphabets are significantly different at 5% level of significance. **</td>
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The present investigation, for the first time reports somatic embryogenesis, plant regeneration and Agrobacterium mediated genetic transformation of somatic embryos in the Indian accessions of Medicago sativa. Identification of regenerative somaclone would help in further biotechnological work in this crop. High frequency of genetic transformation of somatic embryos by the use of 2, 4-D would help in the development of transgenic lucerne with genes of interest.

References