Physical state of the culture medium triggers shift in morphogenetic pattern from shoot bud formation to somatic embryo in *Solanum khasianum*

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Abstract *Solanum khasianum* is a rich source of steroidal alkaloids that are important secondary metabolites with enormous pharmaceutical uses. Development of plantlets from somatic tissues, under in vitro conditions, takes place both through adventitious shoot bud differentiation or somatic embryogenesis (SE) pathway. We observed that the physical state of medium, solid or liquid, determined the regenerant differentiation patterns from root segment explants in *S. khasianum*. In the solidified medium, the root segments developed adventitious shoot buds whereas somatic embryos were regenerated in the liquid medium. Varying gradients from liquid to solid medium were further used to confirm the effect of solidified condition on regeneration pathway. Histological analysis of developing shoot buds and somatic embryos was also performed to confirm their development and differentiation patterns. In

order to further confirm the developmental pathways, qRT-PCR analysis of the marker genes of SE and shoot regeneration was also performed. While *SOMATIC EMBRYOGENESIS RECEPTOR KINASE1* (*SkSERK1*) expression was significantly up-regulated during the early embryogenic stage, the *LATE EMBRYOGENESIS ABUNDANT* (*SkLEA*) protein was found to be highly expressed in the mature embryos. Expression of the *HISTONE DEACETYLASE* (*HDA6*), a repressor of SE related genes, was highly decreased during embryogenesis in the liquid culture. Furthermore, expression of the *ENHANCER OF SHOOT REGENERATION* (*ESR*) gene was comparatively increased during shoot regeneration in the culture using solid medium. Our results point out that the physical state of the medium in *S. khasianum* plays a decisive role in differentiation pattern which was independent of hormonal supplements.

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Introduction

Solanum khasianum (C. B. Clarke) (Family Solanaceae) is an important medicinal plant containing numerous pharmaceutically important steroidal alkaloids, such as solasodine, solanine, solamargine, solasonine, solakhasianine, khasianin etc. which provide an alternative source of steroidal hormones (Sharma et al. 1997; Weissenberg 2001; Jarald et al. 2008; Patel et al. 2013). Solasodine is the major alkaloid from this plant whose role in the promotion of neurogenesis, both under in vitro and in vivo conditions has recently been studied (Lecanu et al. 2011), besides its commercial use as a precursor for the

preparation of oral contraceptives equivalent to diosgenin (Sharma et al. 1997). Berries of this shrub contains the highest concentration of solasodine (~ 1.80 to 3.45%) (Sharma et al. 1997). Steroidal alkaloids of this plant has numerous pharmacological activities such as anticancer, antifungal, hepatoprotective, antiandrogenic, immunomodulatory, antispermatogenic, antishock, antipyretic, antimicrobial, anthelmintic and anti-inflammatory (Jarald et al. 2008; Patel et al. 2013). Although, initial reports of *S. khasianum* are available on micropropagation (Chaturvedi and Sinha 1979) and also on conservation through excised root culture (Chaturvedi et al. 2004) but large scale propagation of this plant is necessary, seeing its versatile importance in traditional system of medicine.

The differentiation through organogenesis and SE is a complex phenomenon and very well described by Thorpe (1993). Each plant or its parts has a particular mode of differentiation, i.e. either through shoot organogenesis or SE but there are many instances where both of these modes of differentiation occur in the same explant albeit induced by one or more factors (Aslam et al. 2013). Both the processes have been reported to occur in the same explant of immature embryos of wheat (He et al. 1990), in a variant of *Helianthus annuus* $\times$ *H. tuberosus* intact adventitious roots (Fambrini et al. 2003) and in *Euphorbia pulcherrima* (Osternack et al. 1999). This shift in morphogenesis may be due to prolonged culture incubation as in citrus callus culture (Chaturvedi and Mitra 1975), or may be due to concentration of growth regulators as in carrot (Kamada and Harada 1979) and *Lens culinaris* Medik (lentils) (Chhabra et al. 2008). SE is an effective way of regeneration in dicots whereby individual somatic cells can undergo differentiation to give rise to genetically uniform plantlets. In a typical somatic embryo, histodifferentiation initiates only by induction of single hormonal signal to form bipolar structure which has the capability to form whole plant (Yang et al. 2013). The development of somatic embryos from particular explants (vegetative tissues) requires critical changes in existing gene expression programme of the tissues to activate the expression of gene networks associated with SE. Regeneration in plants via SE is beneficial and convenient over organogenesis due to several factors such as, single cell origin of somatic embryos, mass propagation with its recurrent properties, large scale easy production of embryos which can be used as synthetic seed, bipolar nature of somatic embryos suitable for direct plant development without extra effort to initiate roots which is strictly required for regeneration via organogenesis (Singh and Chaturvedi 2013; Guan et al. 2016).

Several transcription factors are known to regulate development of somatic embryos in plants. Ectopic expression of *BABY BOOM* (*BBM*), *LEAFY COTYLEDON1* (*LEC1*) and *LEC2* are found sufficient to induce SE

in *Arabidopsis* without exposure to any plant growth regulator (Lotan et al. 1998; Stone et al. 2001; Boutilier et al. 2002). *BBM* regulates *LEC1*, *LEC2* and other *LEC* gene family members like, *FUSCA3* and *ABSCISIC ACID INSENSITIVE3* associated to somatic embryogenesis (Horstman et al. 2017). Some chromatin-remodeling factors like histone deacetylase (*HDA6/HDA19*) which act as transcriptional repressor in eukaryotic system also play important role in SE. They repress embryogenic properties in *Arabidopsis* through repression of *LEC1* genes (Tanaka et al. 2008). *WUSCHEL HOMEODOMAIN 2* (*WOX2*) is expressed during early stages of SE in *Picea abies* (Palo-vaara and Hakman 2008). *WOX2* and *STIMPY-LIKE* (*STPL/WOX8*) regulates the expression of *CUC* genes involved in establishment of cotyledon boundary in the embryos (Lie et al. 2012). Somatic embryogenesis receptor kinase1 (*SERK1*) regulates vegetative to embryogenic transition (Hecht et al. 2001). *SERK1* expression in somatic cell is very crucial to achieve competency to differentiate into embryo (Hecht et al. 2001). Late embryogenesis abundant protein (*LEA*) is known to express at later stages (Roberts et al. 1993; Jin et al. 2014), such as torpedo and cotyledonary stages of SE development. Genes associated to shoot regeneration are also identified in *Arabidopsis* such as *ENHANCER OF SHOOT REGENERATION 1* (*ESR1*) and *ESR2*. *ESR* transcription factors have an AP2/EREBP domain. Expression of *ESR1* and *ESR2* promotes shoot regeneration in *Arabidopsis* (Banno et al. 2001; Ikeda et al. 2006).

In the present study, organogenesis and embryogenesis in root segments of *Solanum khasianum* was achieved under different physical conditions of the same nutrient media. The results obtained were further confirmed by analysing the relative expression of *SkSERK1*, *SkLEA*, *SkHDA6* and *SkESR* in vegetative and embryogenic tissues through qRT-PCR and histological analysis.

Materials and methods

Plant material and culture media

The experimental material of *Solanum khasianum* was used from in vitro-grown multiple shoot cultures. Roots of 20 days-old-plantlets were excised from the healthy shoots and cut into ~ 1 to 2 cm long segments including the root tips. Murashige and Skoog's (Murashige and Skoog 1962) medium was used with some modifications (MSm; concentrations mg l^{-1}) ($1000 \text{ NH}_4\text{NO}_3$, 1500 KNO_3 , $160 \text{ KH}_2\text{PO}_4$, $200 \text{ CaCl}_2 \cdot \text{H}_2\text{O}$, $200 \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 Thiamine HCl, 1.5 Pyridoxine HCl and 1.0 Nicotinic acid) having 3.0% sucrose. In a preliminary experiment, three concentrations of 6-benzyladenine (BA) (0.25 , 0.5 or 1.0 mg l^{-1})

were used in semisolid media to obtain maximum regeneration of shoots from root segments. The pH of all the media was adjusted to 5.2 before adding 0.9% (w/v) agar-agar (Himedia-CR 301). In second experiment, optimized concentration of BA was used with both the liquid and semisolid media. Later, in a separate experiment to further confirm the shift in morphogenetic pattern, different concentrations of agar-agar ranging from 0% (liquid) to 0.5%, 0.6%, 0.7%, 0.8% and 0.9% (semisolid) were used with the same optimized concentrations of BA. After 10, 20 and 30 days incubation, differentiating root segments were observed and photographed under stereoscopic microscope (Leica MZ12.5), while 45-days-old culture were studied using light microscope (Leica S6E).

Statistical analysis

Approximately 15–20 root segments inoculated per petriplate/flask were taken as one replicate. Each experiment consisted of three such replicates for each treatment and all experiments were repeated at least twice. Only the shoot buds with distinctly visible apical dome and leaf primordia were counted and the values of data are presented as mean $\pm$ SE of three replicate cultures and Duncan's multiple range test ($P < 0.05$) were applied to separate the mean using SPSS 16.0.

Growth conditions and data collection

The cultures were incubated at 25 ± 2 °C under a 16 h light and 8 h dark photoperiod at $50\text{--}60 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. The duration of incubation varied from 10 to 45 days according to need of the experiment. Data was recorded after 10, 20 and 30 days of culture incubation and continued till 45 days.

RNA isolation and qRT-PCR analysis

Total RNA was isolated after 10, 20, and 30 days of incubation period from the cut ends of root segments from where callus or shoot buds/embryoid-like structures differentiated. RNA isolation kit (Sigma-Aldrich, Missouri, USA) was used for RNA extraction and treated with the RNase free DNaseI (Ambion, Texas, USA) to remove the residual DNA contaminant. RNA quantity was determined by Nano Drop (ND-1000) spectrophotometer and quality was checked through agarose gel electrophoresis. First strand c-DNA was synthesized by using 3 μg of total RNA with Oligo(dT)₁₈ primers (Thermo Scientific, Lithuania). The qRT-PCR was performed in the Step One Plus Real-time PCR system (Applied Biosystem 7500), using SYBR green for quantification. Expression value was normalized to the expression of reference *UBIQUITIN* gene of *S.*

khasianum. The relative expression of the genes was calculated by $2^{-\Delta\Delta C_t}$ method. List of the primers used in the study is given in Table 1 and sequences of the related genes in the Online Resource 1.

Histology and microscopy

For histological studies, transverse as well as longitudinal sections of differentiating root segments and the embryos were used as experimental material. For section preparation, materials were fixed in FAA solution which contained, Formalin: Glacial acetic acid: 70% alcohol in the ratio of 4:6:9 by volume, respectively, for 2 days. For dehydration and infiltration, ethyl alcohol-xylol series was used (Johansen 1940). The materials were kept in a vacuum oven at 60 °C for 15 min. After proper infiltration, transverse as well as longitudinal sections of differentiating root segments were cut with the thickness of 10–15 μm with the help of microtome (Sartorius-Werke, Göttingen, Germany). Delafield's haematoxylin and safranin were used as differentiating stains. 4 g haematoxylin was dissolved in 30 ml absolute alcohol and mixed in 400 ml saturated solution of ammonium alum, pure glycerine and methyl alcohol (100 ml each) before its use. Safranin stain was prepared by dissolving separately 1 g safranin in 100 ml absolute alcohol and 100 ml MQ water and finally solutions were mixed to each other. Canada balsam was used for mounting the sections for the long term use, whereas temporary slides were prepared in glycerine (10%) using iodine as the stain. Leica MZ12.5 high-performance stereomicroscope was used for anatomical/histological studies.

Results

Shoot organogenesis from root segments

Root tips did not differentiate, instead remained as such and grew only in length. Root segments in the morphogenetic medium on nutrient agar first slightly callused, which was more pronounced at cut ends (Fig. 1a), turned greenish and ultimately differentiated into green shoot buds after an incubation of 15–20 days (Fig. 1b). In a preliminary experiment, shoot organogenesis occurred in all the three BA concentrations (0.25, 0.5 or 1.0 mg l^{-1}) used in the solidified medium. Although maximum number of shoot buds occurred at 1.0 mg l^{-1} BA but that was associated with intervening callus. Therefore, 0.5 mg l^{-1} BA was selected as the optimum treatment for further experiments. Initially 1–2 shoot buds were formed. Thereafter more buds formed successively. Shoot buds were distinguishable under stereomicroscope due to the presence of trichome on primordial structures. Furthermore, shoot buds

Table 1 List of the primers used during study

S. no.	Primer names	Sequences
1.	<i>SkSERK1</i> , forward	5'GTTCCACCACCACCAATTTTC3'
2.	<i>SkSERK1</i> , reverse	5'TAGAGCAGCACCAGCAGCTA3'
3.	<i>SkLEA</i> , forward	5'CGAATCGCCTTTTGACTCTT3'
4.	<i>SkLEA</i> , reverse	5'GCGTATCCACGCCTATGAAT3'
5.	<i>SkUBIQUITIN</i> , forward	5'CCACAGCGGGCAACAGTAA3'
6.	<i>SkUBIQUITIN</i> , reverse	5'TCTCCGTGGTGGTTTTTAGGTT3'
7.	<i>SkESR</i> , forward	5'GACGAGGACAGCGTTTCTGATA3'
8.	<i>SkESR</i> , reverse	5'GCCCATCAAGTCTGAACCAACT3'
9.	<i>SkHDA6</i> , forward	5'TCAGGTTCTACCCCAACTGC3'
10.	<i>SkHDA6</i> , reverse	5'GATGGTATTGGGTGGTGGAG3'

Fig. 1 Shoot organogenesis in excised root segments of *S. khasianum* on solidified agar–agar medium. **a** Callus induction at cut ends after 10 days of culture incubation. **b** Development of shoot buds exogenously embedded in the callus as visible in the enlarged view after 20 days. **c** Shoots at different stages of development after 30 days. **d** Growing shoots after 40 days of culture incubation

were firmly attached to the explant and embedded in the callus cells (Fig. 1c, d).

Somatic embryogenesis from root segments

In contrast to semisolid medium where the root segments regenerated shoot buds, in liquid medium containing same nutrient composition and growth regulators, embryos were differentiated. The root segments after a week of culture incubation showed swelling at their cut ends (Fig. 2a) where the embryos developed and there was secondary embryo formation also. These somatic embryos were formed virtually with no or little callusing of explants and were loosely attached to it during development. Initially,

the embryos were white/creamish in colour but with the development of cotyledon, they turned green in colour. Within 20 days, green coloured embryos visible to the naked eye, developed in liquid media (Fig. 2b). Embryos differentiated both individually as well as in bunch from the root segments. Sometimes these germinating embryos were detached from the root segments (Fig. 2d). Each embryo had a single radicular end and plumular end germinating with root and shoot, respectively (Fig. 2e, f).

The proembryogenic structures were also observed during the embryogenesis i.e.; globular, heart shaped (Fig. 2b, c). The variation in shape, size and number of cotyledons per embryo was common occurrence, and sometimes pluricotyledony also occurred.

Fig. 2 Somatic embryogenesis in excised root segments of *S. khasianum* on liquid medium. **a** Root segments with swollen ends after 10 days of culture incubation. **b** Globular stages of SE differentiated after 20 days of culture incubation. **c** Heart shaped SE visible under stereoscopic microscope after 25 days.

Shift in morphogenetic pattern

To further explore and confirm the shift in morphogenetic pattern, we used various concentrations of agar-agar ranging from 0% (liquid) to 0.5%, 0.6%, 0.7%, 0.8% or 0.9% (semisolid). Rest of the ingredients of the media including BA remained the same. The data has been shown in Table 2. We have observed that root segments cultured on liquid medium (without agar-agar) differentiated into somatic embryos at cut ends. However, in completely semisolid medium (0.9% agar-agar), shoot buds were differentiated. With increasing agar-agar concentration from liquid to semisolid medium, some of the structures formed were neither shoot buds nor somatic embryos but somewhat aberrant structures (Fig. 3). In 0.5 or 0.6% agar-agar, several aberrant type of embryos differentiated from root segments without showing differentiation of shoot meristem at a region opposite to radicular end or with partially differentiated root meristems

d Cotyledonary stage of SE after 30 days of culture incubation. **e** Heart shaped, cotyledonary and germinating somatic embryo. **f** A germinated somatic embryo with well developed root and shoot visible under stereoscopic microscope

(Fig. 3a). The structures formed when explant was cultured on medium containing 0.5% agar, germinated after maturation whereas structure formed from explant cultured on medium containing 0.6% agar, could not germinate into plantlets. In the same way, in the cultures consisting of 0.7 or 0.8% agar-agar, the abnormal structures were more towards shoot buds (Fig. 3b). However, all these structures when subcultured in 0.9% agar-agar, grew into shoots.

Root segments with all the differentiated shoot buds/germinating embryos when subcultured on the basal medium resulted in multiple growing shoots while roots were also formed from the basal portion of the shoots.

qRT-PCR analysis of somatic embryogenesis and shoot regeneration related genes

To further characterize our morphological observation, relative expression pattern of the marker genes, *SkSERK1*,

Table 2 Effect of agar–agar concentrations on shift in morphogenic pattern in *Solanum khasianum* root segments

Morphogenetic pattern	Different concentrations of agar–agar (in g l ⁻¹) at pH 5.2					
	0.0 (liquid)	0.5%	0.6%	0.7%	0.8%	0.9% (solid)
Average number of shoot buds differentiated/plate (mean ± SE)	0.0 ± 0.0 ^e	*2.33 ± 0.34 ^{de}	3.0 ± 0.58 ^d	7.33 ± 0.88 ^c	12.0 ± 1.52 ^b	15.33 ± 1.20 ^a
Average number of somatic embryos differentiated/flask (mean ± SE)	10.66 ± 0.88 ^a	8.0 ± 0.58 ^b	5.66 ± 0.67 ^c	4.0 ± 0.58 ^c	*1.33 ± 0.34 ^d	0.0 ± 0.0 ^d

Culture period 45 days

Value represents the mean ± standard error (SE) followed by the different letters within each row are significantly different according to Duncan's multiple range test (DMRT) at $P < 0.05$

*Differentiated structures were abnormal in appearance and did not germinate

Fig. 3 Some aberrant structures developed in nutrient medium.

a A somatic embryo developed with 0.6% agar–agar in the medium. **b** A shoot bud developed with 0.7% agar–agar in the medium

SkLEA and *HDA6* related to somatic embryogenesis and *ESR* related to shoot regeneration were analyzed. Expression of *SkSERK1* gene was found significantly higher during early induction phase, at proembryogenic stage and even globular stage of somatic embryo development, whereas it was lower at maturation stages of somatic embryos in root segments cultured in the liquid medium (Fig. 4a). In contrast to liquid, expression of *SkSERK1* gene could not be seen at any stage in solid medium (Fig. 4a). Expression of *SkLEA* gene was found significantly higher during 30 days (mature stage) of culture period in liquid medium. Expression of *SkLEA* gene also increased with the culture period in solid medium (Fig. 4b). Expression of *HDA6* (repressor of somatic embryogenesis) was decreased during embryogenesis in liquid medium (Fig. 4c). In contrast to liquid, expression of *ESR* gene was found comparatively higher in the solid medium during shoot induction phases (Fig. 4d).

Histogenesis of shoot bud differentiation

During the process of shoot bud differentiation in excised root segments, mostly the cortical cells were first induced to divide, producing a meristematic tissue at cut ends (Fig. 5a). Several shoot buds differentiated exogenously on this proliferating tissue. Vascular tissue, mostly in the form of nests of tracheary elements, differentiated in the meristematic tissue preceding differentiation of shoot meristems (Fig. 5b). The shoot buds were unipolar structures embedded in the callus cells and firmly attached to the explants through vascular strand and characteristically even the very young shoot buds showed trichomes on the surface (Fig. 5c, d).

Histogenesis of somatic embryo differentiation

Somatic embryos differentiated from the cut ends of root explants with practically no callus. Sometimes a

Fig. 4 Relative expression pattern of the somatic embryogenesis and shoot organogenesis related genes in root segments cultured on medium with agar-agar (0.9%) and without agar. **a–c** Somatic embryogenesis related genes. **d** Shoot organogenesis related gene. 0 days: control root (before treatment), 10 days: callus in solid,

proembryonic and globular stage in liquid; 20 days: shoot bud in solid, globular and heart stage in liquid; 30 days: developed shoot in solid, mature embryo at cotyledonary stage in liquid. Bar represents standard errors (SE) and the different letters shows significance according to Duncan's multiple range test (DMRT) at $P < 0.05$

Fig. 5 Histology of shoot bud differentiation in excised root segments of *S. khasianum*. **a** L.S. of root segments showing proliferated mass of meristematic cells. **b** Exogenous development of shoot buds from mass of proliferated cells and formation of tracheary nests (shown with arrow). **c** L.S. of a differentiated root segment showing different stages of developing primary and secondary shoot buds with well connected vascular strands. **d** A magnified view of a developing shoot bud with apical dome, leaf primordia and vascular strands (shown with arrow)

proembryogenic tissue was formed from the cut ends of root explants. Meristemoids developed in this tissue gave rise to embryos. Embryogenesis was a nonsynchronous

process and different developmental stages, such as globular, heart to mature cotyledonary stage of embryos could be seen anytime. Despite great variability in the

cotyledons, the embryos were bipolar structures having a well formed shoot apical meristem, cotyledons, their own developed vascular supply and root primordia as well (Fig. 6a–d).

Discussion

The availability of oxygen, nutrients and signalling molecules are the important factors for any kind of differentiation. The viability, differentiation and functions of cells depend on oxygen and nutrient availability (Ascough et al. 2004). The differentiation of regenerants is highly dependent on the physical environment of the media. In *S. khasianum*, physical state of culture medium played an important and decisive role in shifting the morphogenetic pattern. The phenomenon of organogenesis and somatic embryogenesis involved organized de novo development which is very well understood in tissue culture (Thorpe 1993). Both the processes, somatic embryogenesis and shoot organogenesis, have been reported to occur in the same explant of immature embryos of wheat (He et al. 1990) and in a variant of *Helianthus annuus* × *H.*

tuberosus intact adventitious roots (Fambrini et al. 2003), and also reported to originate from particular tissue layers or cells within explants in *Euphorbia pulcherrima* (Osternack et al. 1999). In tissue culture, occasionally, there appears a shift in morphogenetic pattern which is induced due to various reasons. Sometimes it is due to prolonged culture incubation as in citrus callus culture (Chaturvedi and Mitra 1975). Concentration of growth regulators also play a very important role for this shift as in carrot, auxins caused development of roots at lower concentration and somatic embryos at higher concentration (Kamada and Harada 1979). Similarly, in *Lens culinaris* Medik (lentils), TDZ induced shoot organogenesis at lower concentration, whereas SE, at higher concentrations (Chhabra et al. 2008). Effect of different types and concentrations of cytokinin on shift in regeneration pathways from shoot regeneration to SE is reported recently in *Metabriggsia ovalifolia* (Ouyang et al. 2016). Different type of PGR induces shoot buds and embryogenesis in *Camellia nitidissima* (Lu et al. 2013). It is also reported in *Phoenix dactylifera*, that in liquid medium higher number of somatic embryos are developed than solidified medium (Ibraheem et al. 2013). In liquid media, the availability of water and nutrients is much more

Fig. 6 Histology of somatic embryo development in excised root segments of *S. khasianum*. **a, b** Different developmental stages of heart shaped embryo. **c** Development of apical dome (AD) and leaf primordia (LP) in a somatic embryo. **d** A well developed SE attached at the cut end of excised root segment with apical dome, growing leaf primordia and a well developed V-shaped vascular strand (shown with arrows)

than solid medium resulting, because of low resistance, in better diffusion and contact between the explant and the nutrient medium (Ascough et al. 2004). In explants of *Catharanthus roseus*, partially merged or submerged in liquid medium, the gaseous exchange occurs without hindrance and the growing tissues use nutrients efficiently (Berthouly and Etienne 2005), whereas, in solid medium, in vitro growth has often been poor as gelling agents like agar reduces water potential in the medium (Ghashghaie et al. 1991). Due to adherence of macro- and micro elements to agar, they are not or less available to growing cells/tissues (Scholten and Pierik 1998). It may be hypothesized that the shift in morphogenetic pattern towards somatic embryogenesis in liquid culture medium in *S. khasianum* might be because of easy accessibility of soluble nutrients and PGRs. There are several reports from different plants where liquid medium proved better for induction of somatic embryogenesis (Bartos et al. 2018; Ibraheem et al. 2013; Castillo et al. 2000).

Developing embryos passes from different intermediate stages and transition of each stage required several physiological and metabolic changes governed by the differential regulation of a set of genes. *SERK1* and *LEA* genes are well reported to express at distinct stages of somatic embryogenesis. *SERK1* gene plays decisive role during development of somatic embryo. It is expressed at early embryogenic stage and its expression lasts up to regeneration (Gruszczynska and Rakoczy-Trojanowska 2011). Expression of *SERK1* in *Arabidopsis* has been found to be critical to obtain competency to differentiate into somatic embryos (Hecht et al. 2001). Additionally, *AtSERK1* overexpressing transgenics of *A. thaliana*, exhibited an enhanced ability for somatic embryogenesis (Hecht et al. 2001). Enhanced expression of *SERK1* during somatic embryogenesis is observed in several plants like carrot (Schmidt et al. 1997), *Dactylis glomerata* (Somleva et al. 2000) and *Arabidopsis* (Hecht et al. 2001). Similarly, we have observed significantly higher expression of *SkSERK1* at early embryo induction stages in root segments cultured on liquid medium which provided molecular evidence for the transition of vegetative tissues into embryogenic tissues. In contrast to this, expression of *SkSERK1* was not enhanced considerably in solid cultures because these cultures followed regeneration program through direct organogenesis (Fig. 4a). Thus, this expression pattern of *SERK1* provided molecular evidence for the shift in morphogenic patterns with the change in physical condition of the medium. *LEA* is reported to be expressed at mature stage of embryo development (Roberts et al. 1993; Jin et al. 2014). We have also observed higher expression of *SkLEA* at mature stage of embryos in liquid culture (Fig. 4b). Expression of *LEA* genes in different tissues has been reported under normal growth conditions

(Gao et al. 2014) and with enhanced expression under the scarcity of water like dehydration, drought, salt stress, etc. (Battaglia and Covarrubias 2013). Expression of *SkLEA* was also observed increasing with the increase in culture period in solid medium which might be due to dehydration of the culture medium with time (Battaglia and Covarrubias 2013). *HDA6* is a chromatin-remodulator which suppresses somatic embryogenesis in *Arabidopsis*. In *Arabidopsis*, treatment with HDA inhibitors enhanced expression of *LEC1*, *FUSCA3*, and *ABSCISIC ACID INSENSITIVE3* genes. Their key role in somatic embryogenesis has been reported (Tanaka et al. 2008). Our results showed that the expression of *HDA6* was reduced in the root segments cultured in liquid medium during somatic embryogenesis whereas in root segments cultured on solid medium, *HDA6* expression was not changed significantly (Fig. 4c). On other hand, we have found that expression of *ESR* was higher in the root segments on solid media which induced shoot organogenesis in root segment of *S. khasianum* (Fig. 4d). It is reported that the overexpression of *ESR1* and *ESR2* enhanced shoot regeneration in *Arabidopsis* (Banno et al. 2001; Ikeda et al. 2006). Relative expression pattern of these SE and shoot organogenesis related genes confirm the shift in morphogenic pattern under two different physical conditions. All the regenerated plants derived from root segments were true-to-type in their morphological characters. On further tracing the origin of regenerants from the root explants, it was mainly found to be the pericycle tissue which represents the exact genetic make-up of the mother tissue (Sunderland 1977). Shoot meristems differentiated from an explant always preceded by meristematic tissue formation from the explant. Thus, the differentiation was never directly from the surface tissue of the explant but shoot buds differentiated exogenously in *S. khasianum* similar to as reported in *Rosmarinus officinalis* (Misra and Chaturvedi 1994). There was formation of tracheary nests in the tissue mass below the differentiating shoot buds in *S. khasianum*. In citrus, before regenerant differentiation, the callus tissue becomes hard, compact, nodular and slow growing accompanied by tracheary tissue differentiation in the deeper zones of the callus (Chaturvedi and Mitra 1975). Shoot buds were having an apical dome which was surrounded by leaf primordia and developed leaves on its flanks. A vascular connection was developed between the newly formed shoot buds and the mother tissue.

It could be concluded that shoot organogenesis and somatic embryogenesis are the two most important and critical morphogenetic phenomenon of differentiation in tissue culture where the shift between the two morphogenetic pattern could occur due to variation in any of the external or internal factors, such as physical condition of the medium, concentration of growth regulators used

externally as well as the internal level of hormones. In the present study, in *S. khasianum* shoot organogenesis occurred in solid medium, whereas this could be shifted to somatic embryogenesis with the use of liquid medium, however, both the media were supplemented with the same nutrient composition.

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Author's contribution SP, PS and PM conceived the original screening and research plans. PM supervised the experiments. SP and PS performed the experiments. SP, PS and PM designed the experiments and analyse the data. The manuscript was written by PS and SP reviewed by PM.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Ascough GD, Fennell CW, van Staden J (2004) The regulation of plant growth and development in liquid culture. *S Afr J Bot* 70(2):181–190
- Aslam J, Srivastava PS, Sharma MP (eds) (2013) Somatic embryogenesis and genetic transformation in plants. Narosa Publishing House, New Delhi
- Banno H, Ikeda Y, Niu QW, Chua NH (2001) Overexpression of *Arabidopsis* ESR1 induces initiation of shoot regeneration. *Plant Cell* 13(12):2609–2618
- Bartos PMC, Gomes HT, do Amaral LIV, Teixeira JB, Scherwinski-Pereira JE (2018) Biochemical events during somatic embryogenesis in *Coffea arabica* L. 3 Biotech. <https://doi.org/10.1007/s13205-018-1238-7>
- Battaglia M, Covarrubias AA (2013) Late embryogenesis abundant (LEA) proteins in legumes. *Front Plant Sci.* <https://doi.org/10.3389/fpls.2013.00190>
- Berthouly M, Etienne H (2005) Temporary immersion system: a new concept for use liquid medium in mass propagation. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Berlin, pp 165–195
- Boutillier K, Offringa R, Sharma VK, Kieft H, Ouellet T, Zhang L, Hattori J, Liu CM, van Lammeren AA, Miki BL, Custers JB, van Lookeren Campagne MM (2002) Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *Plant Cell* 14(8):1737–1749
- Castillo P, Marquez J, Rubluo A, Hernandez G, Lara M (2000) Plant regeneration from callus and suspension cultures of *Valeriana edulis* ssp *procera* via simultaneous organogenesis and somatic embryogenesis. *Plant Sci* 151(2):115–119
- Chaturvedi H, Mitra G (1975) A shift in morphogenetic pattern in *Citrus* callus tissue during prolonged culture. *Ann Bot* 39(4):683–687
- Chaturvedi H, Sinha M (1979) Mass clonal propagation of *Solanum khasianum* through tissue culture. *Ind J exp Biol* 17:153–157
- Chaturvedi H, Sharma M, Sharma A, Jain M, Agha B, Gupta P (2004) In vitro germplasm preservation through regenerative excised root culture for conservation of phytodiversity. *Indian J Biotechnol* 3:305–315
- Chhabra G, Chaudhary D, Varma M, Sainger M, Jaiwal PK (2008) TDZ-induced direct shoot organogenesis and somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris* Medik.). *Physiol Mol Biol Plants* 14(4):347–353
- Fambrini M, Cionini G, Conti A, Michelotti V, Pugliesi C (2003) Origin and development in vitro of shoot buds and somatic embryos from intact roots of *Helianthus annuus* × *H. tuberosus*. *Ann Bot* 92(1):145–151
- Gao C, Liu Y, Wang C, Zhang K, Wang Y (2014) Expression profiles of 12 late embryogenesis abundant protein genes from *Tamarix hispida* in response to abiotic stress. *Sci World J.* <https://doi.org/10.1155/2014/868391>
- Ghashghaie J, Brenckmann F, Saugier B (1991) Effects of agar concentration on water status and growth of rose plants cultured in vitro. *Physiol Plant* 82(1):73–78
- Gruszczynska A, Rakoczy-Trojanowska M (2011) Expression analysis of somatic embryogenesis-related SERK, LEC1, VP1 and NiR orthologues in rye (*Secale cereale* L.). *J Appl Genet* 52(1):1–8
- Guan Y, Li SG, Fan XF, Su ZH (2016) Application of somatic embryogenesis in woody plants. *Front Plant Sci* 7:938
- He D, Yang Y, Bertram J, Scott K (1990) The histological development of the regenerative tissue derived from cultured immature embryos of wheat (*Triticum aestivum* L.). *Plant Sci* 68(1):103–111
- Hecht V, Vielle-Calzada JP, Hartog MV, Schmidt EDL, Boutilier K, Grossniklaus U, de Vries SC (2001) The *Arabidopsis* Somatic Embryogenesis Receptor Kinase 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiol* 127(3):803–816
- Horstman A, Li M, Heidmann I, Weemen M, Chen B, Muino JM, Angenent GC, Boutilier K (2017) The BABY BOOM transcription factor activates the LEC1–ABI3–FUS3–LEC2 network to induce somatic embryogenesis. *Plant Physiol* 175(2):848–857
- Ibraheem Y, Pinker I, Bohme M (2013) A comparative study between solid and liquid cultures relative to callus growth and somatic embryo formation in date palm (*Phoenix dactylifera* L.) cv. Zaghloul. *Emir J Food Agric* 25(11):883–898
- Ikeda Y, Banno H, Niu QW, Howell SH, Chua NH (2006) The ENHANCER OF SHOOT REGENERATION 2 gene in *Arabidopsis* regulates CUP-SHAPED COTYLEDON 1 at the transcriptional level and controls cotyledon development. *Plant Cell Physiol* 47(11):1443–1456
- Jarald EE, Edwin S, Saini V, Deb L, Gupta VB, Wate SP, Busari KP (2008) Anti-inflammatory and anthelmintic activities of *Solanum khasianum* Clarke. *Nat Prod Res* 22(3):269–274
- Jin FY, Hu LS, Yuan DJ, Xu J, Gao WH, He LR, Yang XY, Zhang XL (2014) Comparative transcriptome analysis between somatic embryos (SEs) and zygotic embryos in cotton: evidence for stress response functions in SE development. *Plant Biotechnol J* 12(2):161–173
- Johansen DA (1940) Plant microtechnique. McGraw-Hill Book, New York
- Kamada H, Harada H (1979) Studies on the organogenesis in carrot tissue cultures I. Effects of growth regulators on somatic embryogenesis and root formation. *Z Pflanzenphysiol* 91(3):255–266
- Lecanu L, Hashim AI, McCourtay A, Giscos-Douriez I, Dinca I, Yao W, Vicini S, Szabo G, Erdelyi F, Greeson J, Papadopoulos V (2011) The naturally occurring steroid solasodine induces neurogenesis in vitro and in vivo. *Neuroscience* 183:251–264

- Lie C, Kelsom C, Wu XL (2012) WOX2 and STIMPY-LIKE/WOX8 promote cotyledon boundary formation in Arabidopsis. *Plant J* 72(4):674–682
- Lotan T, Ohto M, Yee KM, West MAL, Lo R, Kwong RW, Yamagishi K, Fischer RL, Goldberg RB, Harada JJ (1998) Arabidopsis LEAFY COTYLEDON 1 is sufficient to induce embryo development in vegetative cells. *Cell* 93(7):1195–1205
- Lu J, Chen R, Zhang M, da Silva JA, Ma G (2013) Plant regeneration via somatic embryogenesis and shoot organogenesis from immature cotyledons of *Camellia nitidissima* Chi. *J Plant Physiol* 170(13):1202–1211
- Misra P, Chaturvedi H (1994) Shoot bud differentiation in leaf segments of *Rosmarinus officinalis* L. In vitro-histology and scanning electron microscopy. *Indian J Exp Biol* 32(4):288–291
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15(3):473–497
- Osternack N, Saare-Surminski K, Preil W (1999) Induction of somatic embryos, adventitious shoots and roots in hypocotyl tissue of *Euphorbia pulcherrima* Willd., ex klotzsch: comparative studies on embryogenic and organogenic competence. *J Appl Bot Angew Bot* 73(5–6):197–201
- Ouyang Y, Chen Y, Lu J, Teixeira da Silva JA, Zhang X, Ma G (2016) Somatic embryogenesis and enhanced shoot organogenesis in *Metabriggsia ovalifolia* W. T. Wang. *Sci Rep* 6:24662
- Palovaara J, Hakman I (2008) Conifer WOX-related homeodomain transcription factors, developmental consideration and expression dynamic of WOX2 during *Picea abies* somatic embryogenesis. *Plant Mol Biol* 66(5):533–549
- Patel K, Singh RB, Patel DK (2013) Medicinal significance, pharmacological activities, and analytical aspects of solasodine: a concise report of current scientific literature. *J Acute Dis* 2(2):92–98
- Roberts JK, DeSimone NA, Lingle WL, Dure L 3rd (1993) Cellular concentrations and uniformity of cell-type accumulation of two LEA proteins in cotton embryos. *Plant Cell* 5(7):769–780
- Schmidt ED, Guzzo F, Toonen MA, de Vries SC (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. *Development* 124(10):2049–2062
- Scholten HJ, Pierik RLM (1998) Agar as a gelling agent: chemical and physical analysis. *Plant Cell Rep* 17(3):230–235
- Sharma RK, Baruah P, Ghosh AC (1997) Effect of transplanting time on incidence of alternaria leaf blight in *Solanum khasianum* Clarke. *Anc Sci Life* 17(1):52–55
- Singh M, Chaturvedi R (2013) Somatic embryogenesis in Neem (*Azadirachta indica* A. Juss.): current status and biotechnological perspectives. Somatic embryogenesis and gene expression. Narosa Publishing House, New Delhi, pp 35–55
- Somleva M, Schmidt E, De VS (2000) Embryogenic cells in *Dactylis glomerata* L. (Poaceae) explants identified by cell tracking and by SERK expression. *Plant Cell Rep* 19(7):718–726
- Stone SL, Kwong LW, Yee KM, Pelletier J, Lepiniec L, Fischer RL, Goldberg RB, Harada JJ (2001) LEAFY COTYLEDON 2 encodes a B3 domain transcription factor that induces embryo development. *Proc Natl Acad Sci USA* 98(20):11806–11811
- Sunderland N (1977) Nuclear cytology. In: Street HE (ed) *Plant tissue and cell culture*. Blackwell, London, pp 177–205
- Tanaka M, Kikuchi A, Kamada H (2008) The *Arabidopsis* histone deacetylases HDA6 and HDA19 contribute to the repression of embryonic properties after germination. *Plant Physiol* 146(1):149–161
- Thorpe TA (1993) In vitro organogenesis and somatic embryogenesis: physiological and biochemical aspects. In: Roubelakis-Angelakis KA, Van Thanh KT (eds) *Morphogenesis in plants*. Springer, Berlin, pp 19–38
- Weissenberg M (2001) Isolation of solasodine and other steroidal alkaloids and sapogenins by direct hydrolysis-extraction of *Solanum* plants or glycosides therefrom. *Phytochemistry* 58(3):501–508
- Yang J, Wu S, Li C (2013) High efficiency secondary somatic embryogenesis in *Hovenia dulcis* Thunb. through solid and liquid cultures. *Sci World J*. <https://doi.org/10.1155/2013/718754>