Advances in Biotechnology

Chapter 2

Limitations in the tissue culture of Indian sandalwood tree (*Santalum album* L.)

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Abstract

The tissue culture of Indian sandalwood (*Santalum album* L.) has been extensively studied, mainly for its rapid propagation technology. *Santalum album* is believed to be the first woody species for which somatic embryogenesis was reported. Despite significant advances, there are still many problems blocking the two basal modes of regeneration, namely, shoot organogenesis and somatic embryogenesis, regardless of other modifications such as protoplast culture or genetic transformation. Without doubt, studies on shoot organogenesis or/and somatic embryogenesis from explants to induce adventitious shoots or somatic embryos of all stages will be continue to be published, but reliable and efficient solutions to the limitations of *S. album* tissue culture are needed. This review highlights these problems, and introduces some physiological and biochemical changes during shoot organogenesis and somatic embryogenesis. Finally, we discuss future research directions for the tissue culture of Indian sandalwood, aiming at a call for more studies that address these problems which hinder the full application of sandalwood tissue culture.

Keywords: *Santalum album*, Indian sandalwood, tissue culture, culture media, callus induction, secondary embryogenesis, maturation of somatic embryos, root formation, plant regeneration

1. Introduction

Indian sandalwood (*Santalum album* L.) belongs to the *Santalum* genus in the Santalaceae, which includes about 18 species [1]. It is believed to be the most valuable species because of a high content of essential oil in heartwood and roots (0.9-8%), which is a higher proportion of α- (41-55%) and β-santalols (16-24%) in essential oil than other sandalwood species [2-6]. So far, Indian sandalwood has been widely planted in India, China and Australia for significant commercial purposes [6]. The trees exhibit great variability and large-scale afforestation provides a feasible way to select superior individual plants [7,8]. *S. album* is a predominantly out-breeding species [9,10], so the traits of offspring are clearly separated. Besides, it takes more than 15 years for Indian sandalwood from planting to harvesting, unlike annual crops which can be selected in time to improve varieties. So, propagating *S. album* trees with desired characters is very important for sustainable development of the sandalwood industry. Although traditional vegetative propagation can be accomplished by grafting, air layering and with root suckers, the production of clones is inefficient, time-consuming and highly dependent on the season [11-13]. The tissue culture of *S. album* is a solution for mass propagation, which is used in limited research on other plant species [6,14,15].

The tissue culture of Indian sandalwood has a history of more than 50 years since 1963 [16], with more than 50 studies published [1,6]. It is believed to be the first woody species for which somatic embryogenesis was reported [1]. Significant advances have been achieved, including a wide range of responsive explants, such as zygotic embryos/endosperms [17-19], hypocotyls [20,21], nodes/internodes [6,22-25], immature leaves [26-29], shoot organogenesis and somatic embryogenesis, cell culture [30], protoplast culture [31-33], and 'synthetic seeds' [34]. Despite all of these, there are still many problems that hinder the full application of tissue culture of Indian sandalwood, such as irregularities in somatic embryos (SEs) [35], low rooting abilities of excised shoots [23,24,36], low survival percentage after regenerated plantlets have been transplanted to the field [20,23,36,37] (Figure 1). So far, the tissue culture of Indian sandalwood has not achieved industrialization. This paper summarizes common problems in the tissue culture of Indian sandalwood, and introduces some physiological and biochemical changes during somatic embryogenesis or shoot organogenesis. Finally, we discuss future research directions of Indian sandalwood tissue culture.

2. Limitations in Indian sandalwood tissue culture

2.1 Callus induction

The main problems during callus induction of Indian sandalwood include the low frequency of callus formation and poor repeatability by different researchers. When Murashige and Skoog (MS) medium was used as the basal medium [38], some researchers used 2,4-dichlorophenoxyacetic acid (2,4-D) alone to induce callus from hypocotyls [35,39] or stem seg-

ments [6,20,35], while other researchers found that callus induced from hypocotyls on callus induction medium (CIM) containing 2,4-D alone could not regenerate any callus [21,40] or that the callus induction efficiency of stem segments was extremely low (20% in [14]; <10% in [41]). In another case, callus was efficiently induced in CIM containing α-naphthaleneacetic acid (NAA) or indole-3-acetic acid (IAA) alone [42]. In contrast, callus often died after subculture [22]. In many cases, researchers used CIM supplemented with combinations of auxins and cytokinins, especially 2,4-D and kinetin (KIN), NAA/IAA and 6-benzyladenine (BA) for callus induction [21,22,25,27,36]. However, the combination differed greatly in different experiments, and the effectiveness and validity of callus induction were questionable [14]. There are many factors that affect callus induction, such as the genotype from which explants are derived, explant age, level of endogenous hormones and culture conditions [43]. Genotype is an important factor that greatly affects callus induction in *S. album*. The choice of clone significantly affected callus development [29]. Spontaneous callus formation for A13 may be controlled by genotype, since the addition of proline, arginine or putrescine did not increase the rate of callus induction [14]. Thidiazuron (TDZ) (0.9-4.5 μM) may be the most efficient plant growth regulator, inducing a high percentage of callus from immature leaves or stem segments, even in different groups of researchers [14,28,44]. In five repeated experiments conducted in September and December of 2015, January, March and July of 2016, we found that callus induction percentage from stem segments of a range of genotypes on MS medium with 2.25-4.54 μM TDZ was consistently high, ranging from 82 to 89% (unpublished data). In another experiment, internodes of nine individuals were used to evaluate the effect of genotype on regeneration mode and callus induction: TDZ was effective for callus induction from different genotypes (Table 1). However, it is worth noting that callus induced by TDZ is usually bright yellow, compact and nodular [28] (Fig. 2A) and with a strong ability to differentiate although the ability to disperse callus is poor, thus making it unsuitable for cell suspension cultures. Relying on the efficiency and stability of TDZ-induced callus, the next research objective is to regulate callus growth to obtain suitable callus for cell suspension culture.

2.2 Asynchronized of somatic embryogenesis

S. album somatic embryogenesis is often observed to be asynchronous. This is partly because of the different level of contact between cultures and media (mainly PGRs) leading to the formation of SEs at different times. It is also because of two kinds of secondary embryogenesis, one in which secondary SEs grow directly on primary SEs and the other in which SEs form callus and then form new SEs, i.e., SEs → callus → SEs [18,20,32,36] (Fig. 2B). Secondary SEs have great potential for rapid propagation [18]. Bapat and Rao (1988) [34] induced and isolated secondary SEs from artificial seeds, which then converted into plantlets. Observations from our experiments showed that secondary somatic embryogenesis was usually induced during the first two subcultures (i.e., during the transfer from SE induction

medium (SEIM) to basal MS medium). In this first step, primary SEs seldom induce secondary SEs, even after three subcultures, which may indicate that secondary embryogenesis is due to the residual effect of PGRs. The frequency of somatic embryogenesis was low but the formation of secondary SEs and abnormal SEs decreased on PGR-free SEIM containing cyanobacterial cell extract compared to SEIM containing PGRs [41]. In another experiment, artificial seeds germinated in basal MS medium, producing secondary SEs in the presence of 2.22 μM BA and 2.85 μM IAA [34], indicating that exogenous PGRs may induce repetitive somatic embryogenesis, although other as yet unknown reasons may exist since the occurrence of secondary SEs in woody plants is common [43,45]. SEs that were first separated then transferred to liquid maturation medium containing abscisic acid (ABA) for a month, and then transferred to solid medium for further conversion into secondary SEs [36].

2.3 Somatic embryo irregularities/abnormalities

During the process of Indian sandalwood somatic embryogenesis, abnormalities in SEs are constantly mentioned, including SEs with no roots or roots with an undeveloped shoot axis, asymmetric cotyledons, monocotyledons, or the fusion of cotyledons [35,46] (Fig. 2C). Different types of abnormal SEs can appear in the same medium and in medium supplemented with different PGRs, which may be caused by endogenous factors during callus differentiation [20], an observation also noted with the culture of protoplasts [32]. In SEIM containing 5.36 μM NAA and 2.21-4.42 μM BA, callus initially induced from nodes or shoot tips by NAA can differentiate into globular, heart-shaped or immature torpedo-shaped SEs but not mature SEs, while immature torpedo-shaped SEs germinate precociously, thus forming abnormal plantlets [42]. In that study, MS medium containing 1.44 μM gibberellic acid (GA_3), 2.46 μM indole-3-butyric acid (IBA) and 2.21 μM BA, various stages of SEs, including cotyledonary SEs, were observed, and precocious germination was completely inhibited. The authors of [42] believed that a high concentration of BA not only decreased the number of SEs in each stage, but also increased the amount of abnormal sSEs, which was consistent with a previous report in 2002 [51]. However, a low concentration of GA_3 and IBA inhibited new SE formation, but promoted SE development into a mature stage, and produced high quality SEs [42]. Other studies showed that mature SEs could germinate in medium supplemented with GA_3, which would otherwise produce many abnormal SEs, thus decreasing the conversion percentage from SEs to plantlets [14,27]. In the absence of exogenous ABA, the frequency of SE maturation was very low, while in optimized medium supplemented with 4.95 μM ABA, the efficiency of somatic embryogenesis increased to 57% [39]. The addition of 3 μM ABA, maltose or 2.5% polyethylene glycol (PEG) to maturation medium increased the proportion of normal SEs [36]. In addition, the use of 1/2MS (macro-elements halved) medium instead of full-strength MS decreased the frequency of abnormal SEs, indicating that a low concentration of mineral elements may promote the formation of normal SEs [47].

Most efforts in sandalwood tissue culture have been to modify the culture conditions to solve the problem of abnormal SE formation. However, studies on the internal factors such as the correlation between SE abnormalities and gene/chromosome variation are rare. It is difficult to detect small chromosomes in somatic cells [23], and chromosome aberrations can occur, e.g., in maize [48].

The following conclusions may be inferred from these studies: 1) the formation of abnormal SEs may be caused by their precocious germination, fusion of many secondary SEs or inhibition by a high concentration of mineral elements in or on the SE radical; 2) one solution is to inhibit premature germination, reduce the frequency of secondary SE formation, induce the maturation of SEs, and use a low concentration of mineral elements to promote root development; 3) ABA, GA_3 or TDZ could be used to obtain mature SEs; IBA might inhibit the occurrence of secondary SEs while GA_3 can promote the germination of SEs.

2.4 Difficulty in rooting of regenerated plantlets

Difficulty with rooting is a major problem in the final stage of shoot organogenesis of Indian sandalwood [21,23,24,27,49,50] (Fig. 2D), and is often encountered in the process of somatic embryogenesis as well [51,52] (Fig. 2E).

A study on the effects of basal media (1/2MS, 1/2 Greshoff and Doy (1972) (GD) medium and 1/2 woody plant medium (WPM) [54]) and different concentrations of KIN (0, 1.16, 2.33, 3.49, 4.65 μM) on rooting showed that 1/2MS with 98.40 μM IBA, 5.71 μM IAA and 3.49 μM KIN was the best combination to induce roots from plantlets [55]. Similarly, we found that MS with 24.60 μM IBA, 0.89 μM BA, and 1.07 μM NAA also induced a high frequency of rooting (74.2%) [6]. We proposed that a low concentration of cytokinins may play a significant role in *S. album* root induction. Many excised shoots remain recalcitrant, and take a long time to form roots (Fig. 2D). When excised shoots were treated with 98.4 μM IBA for different periods, then transferred to the PGR-free medium (1/4MS salts with B_5 [56] vitamins and 2% sucrose) for rooting, IBA treatment period had a significant effect on rooting, and shoots treated for 48 h produced the highest rooting rate (41.67%), root number (2.18 ± 0.06), and root length (3.32 ± 0.67 cm) after 8 weeks [24]. Among four kinds of auxins tested, namely, IBA, NAA, 2-naphthoxy acetic (NOA) and IAA, IBA exhibited the best effect on rooting, and shoots treated with 1230 μM IBA for 30 min showed maximum rooting (50%) in soilrite substrate (peat moss, expanded perlite, and vermiculite; 2:1:1) [24]. Their results showed that treatment with a high concentration IBA had a significant effect on rooting, and that a soil-based substrate was superior to agar medium for rooting, consistent with other observations [6]. Our group also successfully induced roots from excised shoots with a high concentrations of IBA (246 μM) [57].

Mature SEs formed good apical and roots faster in White's medium [58] than in MS me-

dium [22]. Germinated SEs with well-developed shoots and roots could develop into normal plantlets in White's medium with 2.86 μM IAA, indicating significant effects of a low concentration of mineral nutrients [59]. Mature SEs show polarity and develop easily into plantlets with a well-developed radicle and plumule, serving as a method to solve the difficulty of rooting in Indian sandalwood. Another solution to the problem of rooting is *in vitro* micrografting [60], but there is no commercial interest in this method since it requires a seedling to graft a scion.

2.5 Low survival percentage of regenerated plantlets after transplantation into the field

The last step of tissue culture is to transplant regenerated plantlets from sterile culture jars to a field. To date there has been little research on this aspect, mainly because rooting of excised plantlets is difficult, which leads to a shortage of raw materials. One paper [55] studied the effects of different substrates (M1 - volcanic dirt: Purwobinangun, Pakem, Sleman top soils: organic fertilizer = 3:1:1 (v/v/v); M2 - volcanic dirt: Playen, Gunung Kidul top soils: organic fertilizer = 3:1:1; M3 - volcanic dirt: Playen, Gunung Kidul top soils: organic fertilizer = 3:1:1), primary host plants (*Caliandra callotirsus*, *Crotalaria juncea*, and *Capsicum annum*) and their combinations on the survival of transplanted *S. album* plantlets. Results showed that the choice of both substrate and host plant resulted in a slow response to growth and survival of plantlets in a greenhouse. No more than 10% survival of plantlets derived from SEs was shown [23]. The following suggestions were provided to improve this [37]: first, transplanting regenerated plantlets from MS medium to White's medium, then transferring plants from agar medium into sterile substrate, and watering with sucrose-free White's medium to improve root development, and finally placing rooted plants in a mist chamber with high humidity.

3. Biochemical and molecular (DNA/protein) events during Indian sandalwood somatic embryogenesis

Although there are many reports on the culture conditions (especially PGRs) for somatic embryogenesis in *S. album*, the underlying molecular and biochemical events remains unclear. Improving the percentage of SE conversion and the survival percentage of regenerated plants may depend on the elucidation of the metabolic behavior of somatic embryogenesis [61], although related research is still not very profound. The main methods employed have compared the profiles of soluble proteins, amplified DNA fragments, or enzyme (or isozyme) activity in different developmental stages by synchronization or manual separation of cultures. As far as we are aware, only Ca^{2+}-dependent protein kinase (55-60 kD) was identified [62,63] in which Ca^{2+} acted as a secondary messenger in the process of sandalwood somatic embryogenesis while the lack of effective Ca^{2+} (chelated calcium, calcium channel blockers, etc.) significantly reduced the formation of SEs [64]. After 2001, there are no studies that confirm the theories put forward by Anil or Rao.

Fresh callus derived from endosperms in medium containing 2,4-D could partially differentiate into pre-embryogenic masses (PEMs), which developed further into globular SEs in the same medium [17]. In medium containing 2,4-D, however, globular SEs could not develop into bipolar SEs, and bipolar SEs only developed in medium without 2,4-D. In other words, 2,4-D can block somatic embryogenesis in the globular SE stage and bipolar SEs can be obtained in medium without 2,4-D. Based on this, the same authors obtained four stages of cultures, i.e., fresh callus, PEMs, globular SEs, and bipolar SEs. Their experiment showed that during the development process of fresh callus → PEMs → globular SEs → bipolar SEs, the activity of protein kinase gradually increased while the activity of glucosidase and xylanase increased significantly in PEMs → globular SEs and globular SEs → bipolar SEs, indicating that cell wall plasticity was regulated during development [17]. Also, there were differences in protein bands among cultures, indicating possible stage-specific gene expression or post-translational regulation of proteins. The post-translational regulation of proteins was demonstrated in Ca2+-dependent protein kinase [64].

Callus originated from shoot tips and nodes in NAA-fortified media and could differentiate into spherical SEs, heart-shaped SEs and immature torpedo-shaped SEs, but not beyond this stage [42]. Callus could develop into cotyledonary SEs in medium containing 1.44 μM GA_3, 2.46 μM IBA and 2.21 μM BA [42]. Protein analysis of embryogenic tissues by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that some specific proteins (26, 34 and 56 kD) appeared during the period from heart-shaped SEs to torpedo SEs, which may play an important role in the maturation of SEs.

A tissue culture process, namely, compact callus → friable callus → embryo induction stage → embryo mature stage → mature embryos, based on different media, was established [65]. Protein electrophoretic profiles, detection of isozymes (peroxidase, esterase, malate dehydrogenase and shikimate dehydrogenase) and RAPD analysis revealed metabolic changes and genetic variation during somatic embryogenesis. Soluble protein analysis showed that small molecular weight proteins (< 43 kD) were relatively intense in the third stage, which may be a characteristic of embryogenic potential. Changes in the isozyme patterns of these five stages, i.e., the appearance and disappearance of isozyme bands, but without determining the activity of the enzymes, showed that the genes related to these enzymes were differentially activated as development progressed. RAPD patterns in these five stages also showed numerous changes, indicating that under tissue culture conditions, high variability in gene expression took place during somatic embryogenesis.

4. Conclusions and future perspectives

The tissue culture of *S. album* was recently intensively reviewed [1]. The two known regeneration pathways are blocked to some extent (Fig. 1). In addition, since biochemical

and molecular (DNA/protein) events during Indian sandalwood somatic embryogenesis have seldom been exploited, it is necessary to solve key problems mentioned above to achieve the rapid propagation of *S. album*.

Genotype plays an important role in somatic embryogenesis and callus induction [14,29] (Table 1), and provides new solutions but brings new challenges. For example, in most experiments, explants originating from different genotypes or individuals raised from seed were mixed, and the resulting optimal medium may be not useful for select individual plants with desired characters. However, we can use different individual plants to establish different research systems, such as the use of explants to induce somatic embryogenesis easily, allowing for the study of the factors limiting SE germination and the survival of transplanted plantlets. Callus formed easily from individual lines, e.g. line A13 [14], can be employed to detect factors that influence callus differentiation into normal SEs or for screening callus resistant to salts. Since several parameters of regenerated plants derived from somatic embryogenesis differed considerably, including vigor, leaf length and width, phyllotaxy and chlorophyll content [20], it is importance to carry out research that assesses variability in somatic embryogenesis (Fig. 2E). We can use a single genotype (i.e. individual) to construct RAPD/RFPL/SSR fingerprints of different stages of somatic embryogenesis, including regenerated plants, to elucidate the stages that are prone to genetic changes, which will aid the rapid propagation of individuals with excellent and desired characteristics.

In addition, the selection of superior individuals also depends on the development of molecular markers for assessing desired traits. Can we use SaSSy and SaCYP736A167 genes as probes to evaluate the oil production capacity of Indian sandalwood [66,67]? Is it reliable to use peroxidase (POD) activity in bark as a marker of oil yield [68]? These are problems beyond tissue culture which shall be studied as well.

5. Figures

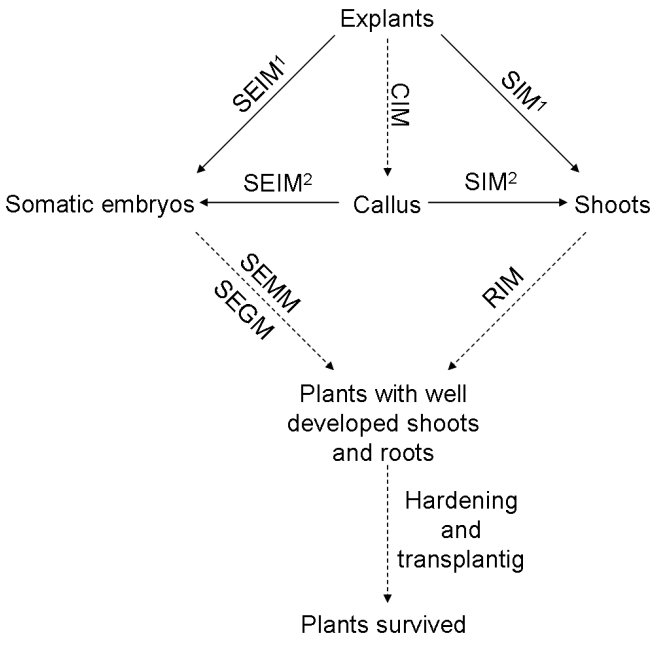

Figure 1: A simplified schematic representing sandalwood tissue culture procedure. SEIM-somatic embryo induction medium; CIM-callus induction medium; SIM-shoot induction medium; SEMM-somatic embryo maturation medium; SEGM-somatic embryo germination medium; RIM-root induction medium. Solid lines indicate easy or effective media while dashed lines indicate ineffective methods or methods with wide variation.

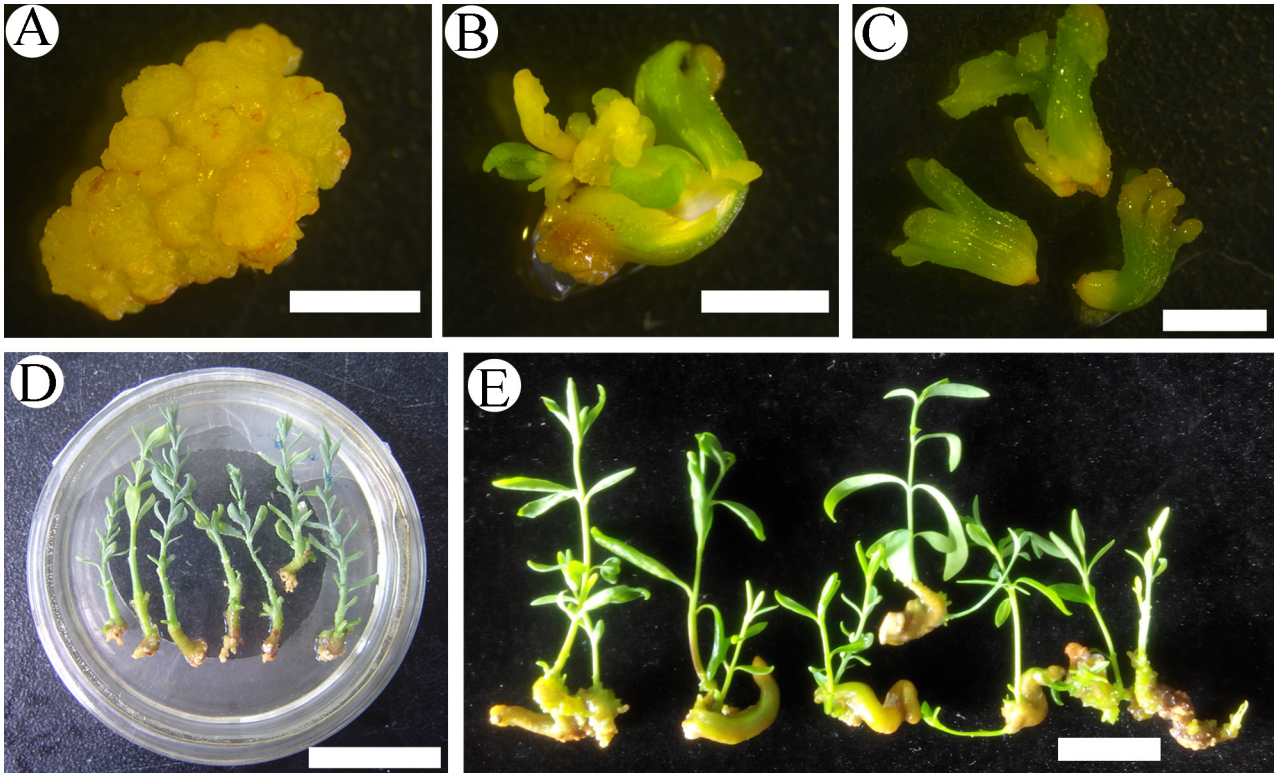

Figure 2: Limitations in tissue culture of Indian sandalwood. (A) Bright yellow, compact and nodular callus was induced from shoot nodes in MS + TDZ (4.54 µM) after one month, bar = 2 mm. (B) Secondary embryos formed directly on the primary somatic embryo, bar = 2 mm. (C) Abnormal embryos with obvious root tip and multi cotyledons, bar = 2 mm. (D) Excised shoots recalcitrant to form roots after 2 months in MS+ IBA (9.84 µM)+ BA (0.89 µM)+ NAA (1.07 µM), bar = 3 cm. (E) Plants raised from somatic embryos with poor roots, the left one showing 3 whorled phyllotaxis different from others, bar = 1 cm.

6. Table

MS media (µM)	Inducing Percentage (%)	Genotype (Sample)								
		1	2	3	4	5	6	7	8	9
BA (2.22)+ NAA (0.27)	Shoots	65	67	62	59	100	100	100	100	100
	Somatic embryos	35	33	38	41	0	0	0	0	0
	Callus	0	0	0	0	0	0	0	0	0
BA (4.44) + NAA (0.54)	Shoots	61	45	58	56	58	100	100	100	100
	Somatic embryos	39	51	44	44	42	34	55	26	45
	Callus	21	19	20	32	11	12	18	12	21
TDZ (4.54) + NAA (0.54)	Shoots	56	35	37	48	100	100	87	91	88
	Somatic embryos	44	65	63	52	89	56	13	9	12
	Callus	87	77	56	67	77	74	78	69	66

7. Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (grant numbers 31470685, 31270720, and 31100498), and the Guangdong Science and Technology project (2015B020231008).

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