Phyton-International Journal of Experimental Botany
DOI: 10.32604/phyton.2020.08971

Tech Science Press

Article

Exogenous Spermidine Promotes Somatic Embryogenesis of *Cunninghamia lanceolata* by Altering the Endogenous Phytohormone Content

Dandan Wang[1,#], Yulin Guo[1,#], Xiaofei Long[1], Yan Pan[1], Dingjie Yang[1], Rui Li[1], Ye Lu[1], Ying Chen[1,2], Jisen Shi[1] and Jinhui Chen[1,*]

[1]Key Laboratory of Forest Genetics & Biotechnology of Ministry of Education of China, Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing, 210037, China

[2]Jiangsu Key Laboratory for Poplar Germplasm Enhancement and Variety Improvement Nanjing Forestry University, Nanjing, 210037, China

[#]These authors contributed equally: Dandan Wang, Yulin Guo

[*]Corresponding Author: Jinhui Chen. Email: chenjh@njfu.edu.cn

Received: 29 October 2019; Accepted: 13 November 2019

Abstract: In order to study how exogenous hormones in *C. lanceolata* (gymnosperm) regulate somatic embryogenesis, we measured the endogenous phytohormones of two genotypes with different somatic embryogenesis efficiency and found that an increase in endogenous concentrations of IAA and ABA may be correlated to more efficient somatic embryogenesis. By applying exogenous spermidine, we found that exogenous hormones may affect somatic embryogenesis efficiency through affecting the endogenous phytohormone content. Based on these results, further studies can be conducted whereby the concentration of exogenous hormones or the levels of endogenous phytohormones by molecular methods are regulated to promote somatic embryogenesis. Our research may benefit the long-term economic output of the forestry industry and lays the foundation to studying the molecular mechanism that controls somatic embryogenesis efficiency.

Keywords: Spermidine; *C. lanceolata*; somatic embryogenesis; endogenous phytohormones

1 Introduction

Due to the long growth cycle of trees, the forestry industry and tree breeding activities benefit greatly from optimized somatic embryogenesis systems. For a large number of species somatic embryogenesis is already successfully applied: for example in *Picea abies* [1], *Pinus massoniana* [2], *Ginkgo biloba* [3], *Liriodendron hybrids* [4] etc. The most suitable explants for induction of somatic embryogenesis may differ for each species. Previous studies have shown that in *Pinus*, somatic embryos can efficiently be induced from immature embryos at the early cotyledon stage, while for *Picea mariana*, slighty older embryos at the cotyledon stage are more effective [5]. Based on morphological observation and physiological research the embryonic development of conifers is divided into four stages: embryogenesis suspensor mass (ESM), early suspensor proembryos (ESP), late suspensor proembryos (LSP) and cotyledon embryos [6]. Filonova et al. [7] used real-time tracking to follow somatic embryogenesis in *Picea abies* and found that proembryogenic masses (PEMs) begin to undergo somatic embryo development after three distinguishable stages (PEM I, PEM II, PEM III), a process that is regulated by plant hormones. The propagation and maintenance of PEMs requires auxin and cytokinin, while further maturation requires ABA [7].

There are many factors that affect somatic embryogenesis, two of which are the plant genotype and the level of endogenous phytohormones [8]. Tang et al. [8] used mature zygotic embryos from eight

different *Pinus taeda* genotypes as explants for embryogenesis. The results showed that, using the same DCR medium and culture conditions, the efficiency of somatic embryogenesis varied wildly between genotypes, with the highest being 81.2%, and the lowest only 9.8%. For other species, similar genotypic variation can be observed, such as in *Picea mariana* [10], *Pinus massoniana* [11], and *Picea glauca* and *P. engelmanii* [12].

It is thought that phytohormones are the key factors controlling plant somatic embryogenesis, and that the level of endogenous phytohormones determines the efficiency of somatic embryogenesis [13]. Chen et al. [14] showed that a high level of endogenous IAA can induce the formation of rice embryonic cells, and that treatment with exogenous IAA can also promote formation of embryogenic cells. Moreover, Michalczuk et al. [15] indicated that auxin levels decrease gradually with the development of somatic embryos. Cytokinin (CTK) is an essential phytohormone in plant growth and development, being involved in cell division, differentiation and morphogenesis [16]. Many studies have shown that cytokinin can induce somatic embryogenesis in plants [17,18]. Upon transferring of the callus of *Lycium barbarum* callus to differentiation medium, the endogenous ABA content increasesd significantly, whereby the first peak appeared as the production of embryogenic cells was observed under the microscope, indicating that ABA is involved in the initiation of embryogenic capacity [19]. Furthermore, the content of ABA in embryonic callus of plant species such as *Cucumis melo* [20] is higher than that of non-embryonic callus. There are few reports of endogenous gibberellin (GA3) regulating somatic embryogenesis and development. Li et al. [21] showed that the endogenous GA3 level in wheat non-embryonic callus is significantly higher than that of embryogenic callus, indicating that GA3 may have negative effects on wheat somatic embryogenesis.

Hormones plays an important role in plant development [13], for example, Melatonin can improve plant stress resistance [22], gibberellic acid plays a protective role in *Petunia* salinity tolerance by improving shoot growth [23], NAA promotes root development in *Lathyrus* [24], researcher treat explants with 6-BA and IAA to induce callus [25]. Studies have shown that the increase of endogenous polyamine content is the premise for somatic embryogenesis in plants; in holm oak, immature somatic embryos contain high levels of spermidine [26]. Kumar et al. [27] noticed that the spermidine level in non-embryonic callus is much lower than that in embryogenic callus in *Coffea canephora*, suggesting that accumulation of spermidine could be key to the formation of embryogenic callus.

For the gymnosperm *C. lanceolata*, the long duration of somatic embryogenic cell masse culture (60-100 days) has always been a challenge, as we found in our laboratory [28]. Therefore, in order to accelerate the process of somatic embryogenesis, we tested the effects of exogenous hormone application on somatic embryogenesis efficiency. We found that exogenous spermidine promotes somatic embryogenesis of *C. lanceolata* and the endogenous phytohormone content was affected by exogenous spermidine. Our results benefit the forestry industry by providing increased production speed and provide further understanding of the function of endogenous phytohormones in somatic embryogenesis. Our findings may function as a basis for further studying the molecular mechanism of somatic embryogenesis.

2 Materials and Methods

2.1 Plant Materials

For all experiments, we used two separate *C. lanceolata* genotypes (01A1, 0183). For callus induction, immature cones we used were collected from the Fujian Yangkou Forest Farm (Fig. 1).

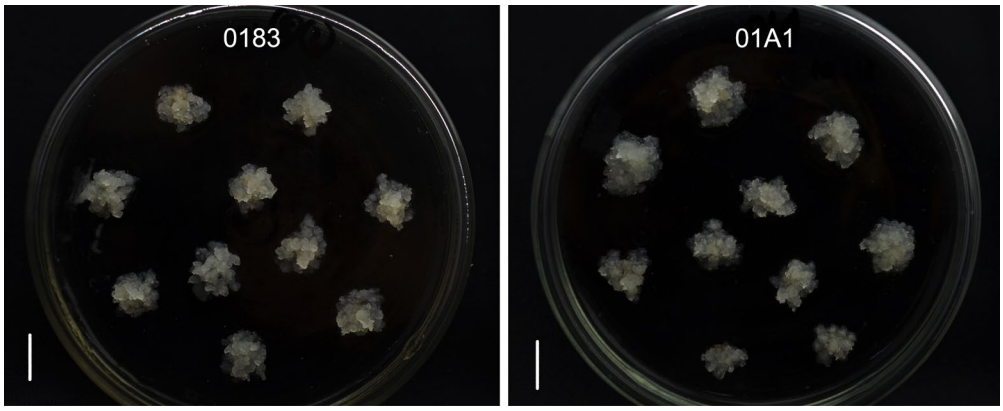

Figure 1: *C. lanceolata* callus derived from two separate genotypes. Scale bar = 1 cm

2.2 Methods

2.2.1 Callus Culture

Based on the callus-inducing medium in our lab (DCR basic medium [29] with content in Tab. 1). We proliferated the callus of the two genotypes for 20 days, cultured in the dark at 23ºC.

Table 1: Callus-inducing medium for *C. lanceolata*

Content	Kinetin	Benzyla-minopurine	Vitamin C	Auxin (2,4-D)	Gluta-mine	Casein hydrolysate	Maltose	Activated carbon	Gelrite
Concen-tration	0.5 mg/L	0.5 mg/L	1 mg/L	1~2 mg/L	0.45 g/L	0.5 g/L	20 g/L	2.5 g/L	2.3 g/L

2.2.2 Somatic Embryo Induction with/without Spermidine

A gradient of spermidine (0, 2, 4, 6, 8 mg/L) was added in our basic somatic embryogenesis-inducing medium (DCR basic medium [29] with content in Tab. 2) to test its effect on embryogenesis efficiency. Each concentration of spermidine was tested in triplicate, placing 6 pieces of callus per dish. Each genotype (0183 and 01A1) was cultured in the dark for 60-100 days, at 23ºC. We used the number of cotyledon embryos per dish formed as a measure method for the efficiency of somatic embryogenesis.

Table 2: Somatic embryogenesis-inducing medium for *C. lanceolata*

Content	Abscisic acid	Gibbere-llin3	Proline	Glutamine	Vitamin C	Casein hydroly-sate	Maltose	Activated carbon	Aspartic acid	Gelrite	Polyethylene glycol (PEG)
Concen-tration	2~8 mg/L	1~5 mg/L	0.2 g/L	0.45 g/L	1 mg/L	0.5 g/L	25 g/L	2.0 g/L	0.2 g/L	2.8 g/L	100-200 g/L

2.2.3 Determination of Endogenous Phytohormone Content

On the one hand, for both genotypes, we collected callus (0.5 g) that was cultured on somatic embryogenesis-inducing medium (without spermidine) for 0 day (0 d) and 45 d (a time after which procotyledon embryo could clearly be observed) to the determine the endogenous concentration of phytohormones IAA, ABA, GA and ZT. Each sample was analyzed in triplicate (Tab. 3). An Enzyme-linked Immunosorbent Assays (ELISA) was used to determine hormone concentrations, according to Bi et al. [30].

Table 3: Sampling from two genotypes for endogenous hormones determination

Genotype	01A1		0183	
Sampling time	0 d	45 d	0 d	45 d
Sampling number	3	3	3	3

On the other hand, for genotype 0183, we collected callus (0.5 g) that was cultured on somatic embryogenic-inducing medium (with spermidine optimum concentration 4 mg/L and without spermidine as control) from 0 day (0 d), 20 days (a time after which somatic embryo initiation could be clearly observed, 20 d) and 30 d (a time after which somatic embryo could be clearly observed, 30 d). Each sample was analyzed in triplicate (Tab. 4) and the measurement method was consistent with above.

Table 4: Sampling from genotype 0183 for endogenous hormones determination

Genotype	0183		
Sampling time	0 d	20 d	30 d
Sampling number	3	3	3

2.2.4 Data Analysis

Statistical tests, such as one-way ANOVA and multiple-comparison tests, were performed using SPSS 25 (https://www.ibm.com/analytics/spss-statistics-software), and charts were drawn using GraphPad Prism 7 (https://www.graphpad.com/data-analysis-resource-center/).

3 Results

3.1 Endogenous Hormone Content Affects C. lanceolata somatic embryogenesis

As we mentioned previously, endogenous phytohormones have been shown to be involved in somatic embryogenesis. We therefore examined whether the endogenous hormone content (IAA, ABA, GA, ZT) of two separate *C. lanceolata* genotypes (01A1, 0183) with different somatic embryogenesis efficiencies could be correlated with the amount of embryos formed.

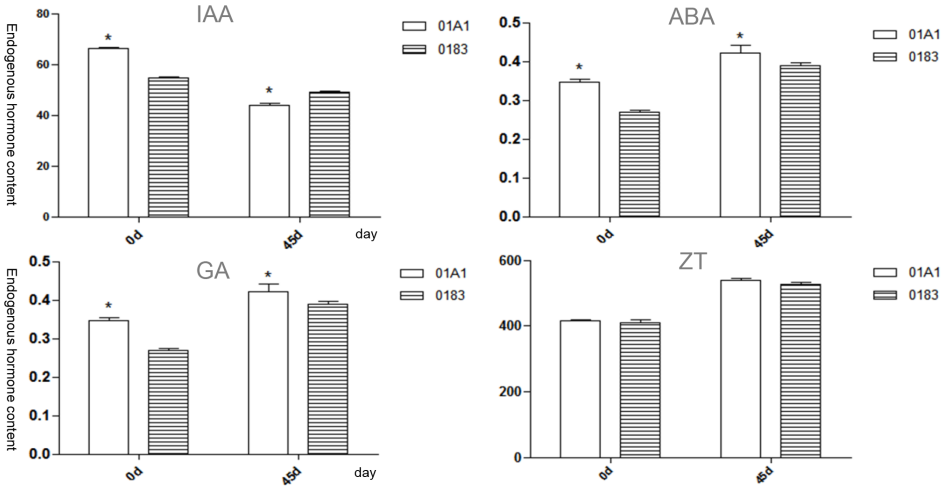

Figure 2: Endogenous IAA, ABA, GA and ZT levels in both *C. lanceolata* genotypes at 0 and 45 days. Statistical differences were calculated using a one-way ANOVA test, * $P < 0.05$, error bars represent SD

Callus derived from genotype 01A1 has a higher somatic embryogenesis efficiency based on our previous observations, and its endogenous GA and ABA contents are significantly higher than in the 0183 genotype at the 0 d and 45 d after somatic embryo induction, meanwhile, the IAA content in 01A1 genotype is significantly higher than in 0183 genotype (0 d), but then significantly lower than in 0183

genotype (45 d) (Fig. 2). By contrast, we could not detect significant differences in ZT level between the two genotypes (Fig. 2). Consistent with previous studies, we observed an overall decrease in IAA content and an increase in ABA content during the process of somatic embryogenesis [15].

These findings indicate that the level of endogenous phytohormones, especially IAA (high in the beginning and decrease during somatic embryogenesis), GA (increasing during somatic embryogenesis) and ABA (increasing during somatic embryogenesis), varies during somatic embryogenesis and suggests that they may have an effect on the embryogenesis efficiency of different genotypes.

3.2 Exogenous Spermidine Promotes C. lanceolata somatic embryogenesis

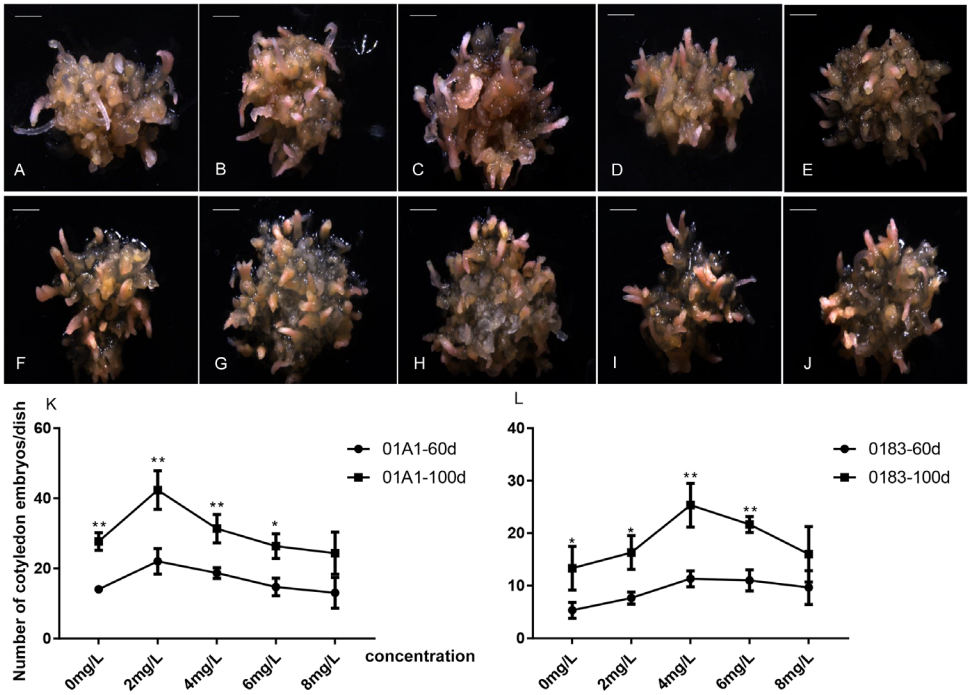

Figure 3: Number of cotyledonous embryos produced at different concentrations of spermidine. A-E, somatic embryogenesis of genotype 0183 at increasing concentrations (0 mg/L, 2 mg/L, 4 mg/L, 6 mg/L, 8 mg/L) of spermidine. F-J, somatic embryogenesis of genotype 01A1 at increasing concentrations (0 mg/L, 2 mg/L, 4 mg/L, 6 mg/L, 8 mg/L) of spermidine. Scale bar = 5 mm. K-L, number of cotyledonous embryos produced per genotype at each concentration of spermidine used. Statistical significance was determined using a one-way ANOVA test, ** $p < 0.01$, * $p < 0.05$, error bars represent SD

In order to improve the efficiency of somatic embryogenesis in *C. lanceolata*, we treated callus from two genotypes (01A1, 0183) with a gradient (0-8 mg/L) of exogenous spermidine. We found that for both genotypes, spermidine enhances somatic embryogenesis efficiency, with each genotype having its own optimal concentration at which embryo production peaks (Fig. 3). In all cases, maintaining the culture for an extended duration of time (up to 100 days) led to the production of a significantly increased number of embryos with a gradient (0-6 mg/L) of exogenous spermidine (Fig. 3).

These findings illustrate that, although spermidine promotes somatic embryogenesis, the optimum concentration to be used will have to be determined for each new genotype, since too high concentrations are ineffective.

3.3 Exogenous Spermidine Promotes C. lanceolata somatic Embryogenesis by Affecting Endogenous Phytohormone Levels

We speculated that exogenous spermidine exerts its effects by affecting the content of endogenous

phytohormones [15]. To verify this hypothesis, we selected genotype 0183 for endogenous hormone monitoring at successive timepoints. We found that all four endogenous phytohormones (IAA, ABA, GA, ZT) show increased levels after 20 days of exogenous spermidine treatment (Fig. 4).

It is worth noting that after 20 days, IAA content significantly increases in the spermidine treated callus compared to the control (non-treated) and shows a significantly lower level than control after 30 days. Meanwhile, GA content shows a trend of first increasing and then decreasing within 30 days, a trend that appears similar to the changes observed in IAA content. By contrast, ABA and ZT show an exclusively increasing trend (Fig. 4), which indicating that after the initiation of somatic embryos, their development may require less IAA and GA, but more ABA and ZT.

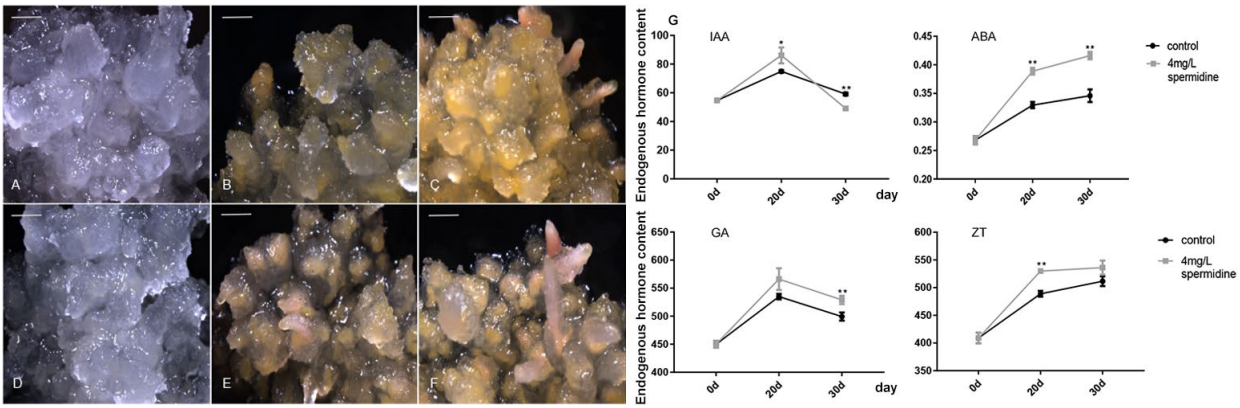

Figure 4: Spermidine affects endogenous hormone content during somatic embryogenesis. A-C, somatic embryogenesis of genotype 0183 (non-spermidine treatment). D-F, somatic embryogenesis of genotype 0183 (4 mg/L spermidine treatment). Scale bar = 2 mm. G, Endogenous hormone content of treated and untreated callus. Significance was determined using a one-way ANOVA test, ** $p < 0.01$, * $p < 0.05$, error bars represent SD

4 Discussion

Based on previous research into how endogenous phytohormones may affect somatic embryogenesis [14,16,19,21], we examined whether differences in hormone content might explain why two separate *C. lanceolata* genotypes have distinct somatic embryogenesis efficiencies. Our results showed that the endogenous IAA and ABA levels of the genotype having high somatic embryogenesis efficiency are increased compared to the less well responding genotype. During somatic embryogenesis, for high somatic embryogenesis efficiency genotype (01A1), IAA levels gradually decrease, consistent with the results previously obtained by Michalczuk et al. [15] in carrot. Moreover, to improve the efficiency of somatic embryogenesis in two genotypes of *C. lanceolata*, exogenous spermidine was treated in both callus, and results shows at a certain concentration of exogenous spermidine promotes somatic embryogenesis, which agrees with the research of Monteiro et al. [32]. As we show, spermidine presumably acts by increasing the levels of several endogenous phytohormones, findings consistent with results obtained in *Araucaria angustifolia* [31].

Based on the results above, a series of experiments could be performed to improve the somatic embryogenesis efficiency of *C. lanceolata*. One might overexpress ABA related genes, knock-down IAA related genes or treat callus with exogenous ABA, GA and/or ZT. However, it should be noted that the content of exogenous hormones between different genotypes will not be identical. Furthermore, the molecular mechanism of how each exogenous hormone affects somatic embryogenesis, in *C. lanceolata* or other species, can be interesting topics for future research. Meanwhile, studies have shown that plant hormone can regulate heavy metal stress tolerance in plants [33]. There are a lot of heavy metal pollution, such as Aluminum [34,35] and Cadmium [22]. Thus, it may be a good research direction for exogenous hormones to resist heavy metals in plants.

In summary, our experiments provide a good foundation for the improvement of somatic embryogenesis protocols used by the forestry industry of *C. lanceolata*. Further research on the molecular mechanism in the future may lay a theoretical foundation for the somatic embryogenesis of gymnosperms.

Acknowledgement: This research was supported by Foundation of Jiangsu forestry bureau (LYKJ[2017]42), Key research and development plan of Jiangsu Province (BE2017376), the Nature Science Foundation of China (31770715), the Qinglan project of Jiangsu province, Priority Academic Program Development of Jiangsu Higher Education Institutions, Natural Science Foundation of Jiangsu Province (BK20181176) and the Joint Fund of the Natural Science Foundation of China and the Karst Science Research Center of Guizhou Province (Grant No. U1812401). The authors wish to thank the editor and reviewers for their helpful comments and suggestions.

Conflicts of Interest: The authors declare no conflict of interest.

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