'Ningqi 8' Post-establishment Imprint of Somatic Embryogenesis System

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Abstract

Using young leaves of the new Ningxia goji berry (Lycium barbarum) variety 'Ningqi 8' as explants, this study investigated the effects of hormone combinations and additives on somatic embryogenesis induction, somatic embryo proliferation, germination, and plant regeneration of 'Ningqi 8' , aiming to establish an efficient and stable somatic embryogenesis system. The results showed that through orthogonal analysis of 6-BA, 2,4-D, and IAA, the optimal hormone combination for somatic embryo induction in 'Ningqi 8' was screened out: 6-BA 1.0 $mg \cdot L^{-1}$ + 2,4-D 0.3 $mg \cdot L^{-1}$ + IAA 0.4 $mg \cdot L^{-1}$, achieving a somatic embryo induction rate of 88.67%. Range analysis comparing the effects among various hormones revealed that 6-BA had the most significant influence on somatic embryo induction. Only when an appropriate ratio of high-concentration auxin to low-concentration cytokinin was applied could morphologically normal and numerous somatic embryos of 'Ningqi 8' be induced. In somatic embryo proliferation culture, excessively high concentrations of 6-BA easily led to vitrification, which was detrimental to the proliferation and growth of 'Ningqi 8' somatic embryos. With increasing hormone concentrations, the somatic embryo proliferation multiplier increased, but the vitrification rate also became higher. Comprehensive analysis identified the optimal somatic embryo proliferation culture for 'Ningqi 8' as 6-BA 0.4 $mg \cdot L^{-1}$ + NAA 0.6 $mg \cdot L^{-1}$. When IBA 0.3 $mg \cdot L^{-1}$ + GA_3 0.4 $mg \cdot L^{-1}$ + sucrose 10 $g \cdot L^{-1}$ was added, the somatic embryo germination rate of 'Ningqi 8' was the highest, reaching 89.17%. Under conditions of GA_3 addition and low sucrose concentration, the germination of mature somatic embryos could be promoted. The degree of influence on somatic embryo germination of 'Ningqi 8' followed the order: IBA > sucrose > GA_3. Activated charcoal could effectively improve the plant regeneration rate from 'Ningqi 8' somatic embryos, while also promoting root development in germinated somatic embryos. When IBA 0.1 $mg \cdot L^{-1}$ + KT 0.4 $mg \cdot L^{-1}$ + activated charcoal 1 $g \cdot L^{-1}$ was applied, the plant regeneration from somatic embryos of 'Ningqi 8' achieved the best results, with a somatic embryo regeneration rate of

91.67%.

Full Text

Abstract

In order to establish a high-frequency and stable somatic embryogenesis system for the new wolfberry cultivar 'Ningqi 8' , we investigated the effects of hormone combinations and additives on somatic embryo induction, proliferation, germination, and plant regeneration using young leaves as explants. Through orthogonal analysis of 6-BA, 2,4-D, and IAA, the optimal hormone combination for somatic embryo induction was identified as 6-BA 1.0 $mg \cdot L^{-1}$ + 2,4-D 0.3 $mg \cdot L^{-1}$ + IAA 0.4 $mg \cdot L^{-1}$, achieving an induction rate of 88.67%. Range analysis revealed that 6-BA exerted the most significant influence on somatic embryogenesis. Only when high auxin concentrations were appropriately combined with low cytokinin concentrations could numerous morphologically normal somatic embryos be induced. During proliferation culture, excessive 6-BA concentrations readily caused vitrification, which was detrimental to somatic embryo growth. Although proliferation rates increased with higher hormone concentrations, so did vitrification rates. The optimal proliferation medium was determined to be 6-BA 0.4 $mg \cdot L^{-1}$ + NAA 0.6 $mg \cdot L^{-1}$. The highest germination rate of 89.17% was achieved with IBA 0.3 $mg \cdot L^{-1}$ + GA_3 0.4 $mg \cdot L^{-1}$ + sucrose 10 $g \cdot L^{-1}$. Under these conditions of GA_3 supplementation and low sucrose concentration, mature somatic embryo germination was promoted, with IBA showing the greatest influence, followed by sucrose and then GA_3. Activated carbon effectively enhanced plantlet regeneration rates and promoted root development in germinating embryos. The optimal regeneration combination was IBA 0.1 $mg \cdot L^{-1}$ + KT 0.4 $mg \cdot L^{-1}$ + activated carbon 1 $g \cdot L^{-1}$, yielding a regeneration rate of 91.67%.

Keywords: 'Ningqi 8' , somatic embryogenesis, somatic embryo induction and proliferation, somatic embryo germination, plant regeneration

Introduction

Lycium barbarum (wolfberry), a perennial branching shrub belonging to the Solanaceae family, possesses significant medicinal and ecological value. As research into wolfberry' s pharmaceutical and health benefits has deepened, cultivation areas have expanded annually. However, existing varieties with single-purpose applications struggle to meet the diverse demands of processing enterprises, necessitating the development of new cultivars with broader utility (He et al., 2006). While hybrid breeding represents the earliest and most conventional approach, its breeding cycle is lengthy. Integrating biotechnology with conventional breeding offers novel technical pathways for wolfberry cultivar development. Wolfberry biotechnology research began in the early 1980s and has encompassed various aspects of plant cell engineering, including rapid

propagation of superior varieties, embryo culture, protoplast culture, and haploid/polyploid breeding (Ren et al., 2007).

Somatic embryos offer unique advantages for plant cell engineering, including single-cell origin, rapid propagation, uniform genetic background, and chromosomal stability. They constitute a critical component for genetic transformation, germplasm preservation, artificial seeds, and other biotechnological applications, while also serving as the foundation for suspension cell line establishment, cell mutant induction and screening, and protoplast culture and fusion. Establishing a somatic embryogenesis system for wolfberry will provide an excellent platform for biotechnology-based breeding and accelerate cultivar improvement efforts.

Previous studies have laid important groundwork. Cao et al. (1997) induced embryoid formation from suspension-cultured pith tissues of 'Ningqi 1' . Ma et al. (2005) investigated somatic embryogenesis from immature embryos of 'Ningqi 2', examining hormone, light, and temperature effects. Wang et al. (2014) established high-frequency somatic embryogenesis systems using seeds from 'Ningqi 1' , 'Ningqi 2' , 'Ningqi 4' , and Xinjiang wolfberry. However, due to genotypic differences, optimal conditions vary substantially among cultivars. 'Ningqi 8' , a new large-fruit wolfberry cultivar obtained through natural selection, exhibits vigorous growth, large fruit size (longitudinal diameter up to 4.3 cm—double that of 'Ningqi 1'), and high nutritional value (Nan et al., 2014). Despite these superior characteristics, no studies on somatic embryogenesis of 'Ningqi 8' have been reported. Establishing this system would provide a new pathway for wolfberry cultivar improvement, enabling rapid, large-scale propagation independent of seasonal constraints and facilitating industrial production of this superior variety.

Plant growth regulators play crucial roles throughout somatic embryogenesis. 2,4-D is commonly used for explant dedifferentiation but must be reduced or removed promptly to allow normal embryogenic cell development (Merkle, 2000). Combinations of IBA and KT primarily promote somatic embryo maturation, while GA_3 shows limited effect on induction but significantly enhances maturation. Activated carbon adsorbs toxic metabolic byproducts, promoting embryo differentiation and development. Different hormones serve distinct functions, and optimal combinations vary by explant type. Although most domestic research has utilized endosperm, seeds, cotyledons, and hypocotyls for wolfberry somatic embryogenesis, leaf explants offer greater convenience and better preserve genetic characteristics. This study investigates the effects of various additives on somatic embryogenesis in 'Ningqi 8' leaves to establish an efficient system and lay the foundation for wolfberry biotechnology breeding.

Materials and Methods

1.1 Plant Material

Young leaves from shoot tips of the new wolfberry cultivar 'Ningqi 8' were collected from the Wolfberry Germplasm Repository at Ningxia Forestry Institute.

This cultivar received national plant variety protection in 2012 and was certified as an improved forest variety by Ningxia Autonomous Region in 2015.

1.2 Experimental Methods

1.2.1 Somatic Embryo Induction Healthy, pest-free young leaves were washed under running tap water for 30 minutes. Under aseptic conditions, leaves were surface-sterilized with 75% ethanol for 30 seconds, rinsed 3–4 times with sterile water, treated with 0.1% $HgCl_2$ solution for 3–4 minutes, and rinsed again 3–4 times with sterile water. Leaves were then cut into 0.5 cm × 0.5 cm pieces and inoculated onto induction medium.

An $L_9(3^4)$ orthogonal design was employed to investigate the effects of 6-BA (0.5, 1.0, 1.5 $mg \cdot L^{-1}$), 2,4-D (0.3, 0.6, 0.9 $mg \cdot L^{-1}$), and IAA (0.2, 0.4, 0.6 $mg \cdot L^{-1}$) on somatic embryo induction, comprising nine treatments. The basal medium consisted of MS + sucrose 30 $g \cdot L^{-1}$ + agar 6 $g \cdot L^{-1}$. Each treatment included 10 replicate vessels with 5 explants each, repeated three times. After 40 days of culture at 23–25 °C under 16 $h \cdot d^{-1}$ photoperiod (2000 lx), somatic embryo induction rates and growth status were recorded.

1.2.2 Somatic Embryo Proliferation A two-factor completely randomized design examined 6-BA (0.4, 0.8 $mg \cdot L^{-1}$) and NAA (0.2, 0.4, 0.6 $mg \cdot L^{-1}$) effects on embryo proliferation, comprising six treatments. Normal 'Ningqi 8' somatic embryo clusters were cut into 0.2 cm × 0.3 cm pieces and inoculated onto proliferation media (basal medium: MS + sucrose 30 $g \cdot L^{-1}$ + agar 6 $g \cdot L^{-1}$). Each treatment included 10 vessels with 5 embryo clusters each, repeated three times. After 40 days, proliferation rates and vitrification rates were recorded.

1.2.3 Somatic Embryo Germination The effects of IBA, GA_3, and sucrose on embryo germination were investigated using an $L_9(3^4)$ orthogonal design: IBA concentration (0.1, 0.2, 0.3 $mg \cdot L^{-1}$), GA_3 concentration (0.2, 0.4, 0.6 $mg \cdot L^{-1}$), and sucrose (10, 20, 30 $g \cdot L^{-1}$) with MS as basal medium. Mature embryos were inoculated onto test media (10 vessels per treatment, 4 embryos per vessel, three replicates). After 30 days, germination rates and growth status were recorded.

1.2.4 Plantlet Regeneration Eight treatments were established combining IBA (0.1, 0.3 $mg \cdot L^{-1}$) and KT (0.2, 0.4 $mg \cdot L^{-1}$) with and without 1 $g \cdot L^{-1}$ activated carbon . The basal medium was MS + sucrose 30 $g \cdot L^{-1}$ + agar 6 $g \cdot L^{-1}$. Embryos with radicles were inoculated (10 vessels per treatment, 4 embryos per vessel, three replicates). After 25 days, plantlet regeneration rates, growth status, and shoot proliferation coefficients were recorded.

1.2.5 Data Processing and Statistical Analysis The following formulas were used: - Somatic embryo induction rate (%) = (Number of leaf explants forming embryos / Total inoculated leaves) × 100 - Proliferation multiple =

(Number of new embryos produced / Number of inoculated embryos) - Vitrification rate (%) = (Number of vitrified embryos among newly proliferated embryos / Total tested embryos) × 100 - Germination rate (%) = (Number of embryos forming roots / Total embryos for germination test) × 100 - Plantlet regeneration rate (%) = (Number of plantlets with complete roots and shoots / Total tested embryos) × 100 - Shoot proliferation coefficient = (Number of shoots produced / Number of inoculated embryos)

Data were analyzed using DPS software with Duncan' s multiple range test for significance.

Results

2.1 Effects of Hormones on Somatic Embryo Induction

Based on K-value analysis in , optimal levels were 6-BA at 1.0 mg · L^{-1}, 2,4-D at 0.3 mg · L^{-1}, and IAA at 0.4 mg · L^{-1}, yielding the optimal combination: 6-BA 1.0 mg · L^{-1} + 2,4-D 0.3 mg · L^{-1} + IAA 0.4 mg · L^{-1} (Treatment 4). This treatment produced the highest induction rate of 88.67%, confirming its superiority. The R-value (range) indicated factor significance, with 6-BA showing the largest R-value and thus the most significant effect on 'Ningqi 8' somatic embryogenesis. The influence hierarchy was 6-BA > IAA > 2,4-D.

2.2 Morphological Observation of Somatic Embryo Development Stages

During induction, leaves gradually expanded and arched after 10 days. By day 15, numerous dense protrusions appeared at cut surfaces as green globular structures [FIGURE:1: A]. After 20 days, irregular cell masses formed. By day 25, proliferating cell masses enveloped the entire explant. Microscopic observation revealed well-developed globular [FIGURE:1: B], heart-shaped [FIGURE:1: C], and torpedo-stage embryos [FIGURE:1: D]. Globular embryos were macroscopically visible due to their larger size. Multiple developmental stages coexisted within the same embryo cluster, predominantly globular embryos, with torpedo-stage and rapidly developing cotyledonary embryos exhibiting green or purple-red shoots [FIGURE:1: E]. As embryos matured, numerous radicles emerged from germinating embryo clusters [FIGURE:1: F].

2.3 Effects of 6-BA and NAA on Somatic Embryo Proliferation

As shown in , when 6-BA concentration remained constant, proliferation multiples increased with NAA concentration. Treatment 6 (6-BA 0.8 mg · L^{-1} + NAA 0.6 mg · L^{-1}) achieved the highest proliferation multiple (13.22) but also the highest vitrification rate. Excessive 6-BA concentrations caused vitrification, impairing embryo proliferation. Treatment 3 (6-BA 0.4 mg · L^{-1} + NAA 0.6 mg · L^{-1}) yielded a proliferation multiple of 12.28, which was not significantly different from Treatment 6 at P=0.05, but with significantly lower vitrification

rates. Therefore, the optimal proliferation medium was 6-BA 0.4 mg · L^{-1} + NAA 0.6 mg · L^{-1} (Treatment 3).

2.4 Effects of Hormones and Sucrose on Somatic Embryo Germination

Germination results are presented in . Treatment 8 achieved the highest germination rate (89.17%) with significant differences from other treatments. K-value analysis indicated optimal levels of IBA 0.3 mg · L^{-1}, GA_3 0.4 mg · L^{-1}, and sucrose 10 g · L^{-1}, confirming the optimal combination as IBA 0.3 mg · L^{-1} + GA_3 0.4 mg · L^{-1} + sucrose 10 g · L^{-1} (Treatment 8). IBA exhibited the largest R-value, establishing it as the dominant factor influencing germination, with the hierarchy: IBA > sucrose > GA_3.

2.5 Effects of Hormones and Activated Carbon on Plantlet Regeneration

Analysis of demonstrated that activated carbon supplementation significantly enhanced plantlet regeneration rates, root number, root length, and shoot proliferation coefficients compared to controls. At IBA 0.1 mg · L^{-1}, KT 0.4 mg · L^{-1} outperformed KT 0.2 mg · L^{-1}, whereas at IBA 0.3 mg · L^{-1}, KT 0.2 mg · L^{-1} was superior. This indicates that optimal auxin-cytokinin ratios, rather than simply higher concentrations, promote regeneration. The optimal combination was IBA 0.1 mg · L^{-1} + KT 0.4 mg · L^{-1} + activated carbon 1 g · L^{-1} (Treatment 4), achieving a maximum regeneration rate of 91.67%.

Discussion and Conclusion

Plant growth regulators are the most active factors in somatic embryogenesis, with distinct types and concentrations required at different developmental stages. Our results demonstrate that appropriate ratios of high auxin to low cytokinin concentrations are essential for inducing numerous morphologically normal 'Ningqi 8' somatic embryos. The highest induction rate (88.67%) was achieved with 6-BA 1.0 mg · L^{-1} + 2,4-D 0.3 mg · L^{-1} + IAA 0.4 mg · L^{-1}, though excessive 6-BA inhibited embryogenesis. The hormone influence hierarchy (6-BA > IAA > 2,4-D) aligns with findings by Zhang et al. (1996), who reported maximum embryogenic callus formation in wolfberry young embryos cultured on medium containing 6-BA 0.5–1.0 mg · L^{-1} and IAA 0.5 mg · L^{-1}. Luo et al. (2016) similarly found that 6-BA and NAA combinations maximized embryogenic callus differentiation frequency in wolfberry anther culture, showing comparable hormone type and concentration preferences.

Most plant species require 2,4-D for embryogenic cell induction in vitro, as it plays a crucial role in regulating endogenous auxin balance (Zhang, 2007). Zhang et al. (2016) reported optimal wolfberry anther embryogenesis at 2,4-D 0.2 mg · L^{-1}, similar to our optimal 0.3 mg · L^{-1}, with minor variations attributable to cultivar and explant differences. While previous wolfberry somatic embryogenesis studies utilized endosperm, seeds, cotyledons, anthers, pollen,

and hypocotyls, our leaf explant approach offers greater convenience, abundance, and year-round availability. Under our optimized three-hormone combination, induction rates substantially exceeded those reported for other explant types.

Proliferation produces abundant morphologically normal embryos for subsequent germination and regeneration studies. Our investigation of 6-BA and NAA ratios revealed that while 6-BA 0.8 mg·L^{-1} + NAA 0.6 mg·L^{-1} maximized proliferation capacity, it also caused severe vitrification. Reducing 6-BA to 0.4 mg · L^{-1} with NAA 0.6 mg · L^{-1} maintained strong proliferation while significantly decreasing vitrification. This finding corroborates Pan et al. (2018), who reported that appropriate NAA and 6-BA concentrations significantly enhanced proliferation in resistant *Pinus densiflora*. However, vitrification occurred across all treatments in 'Ningqi 8' , warranting further investigation. Additionally, while our explants encompassed various developmental stages, the optimal stage for proliferation requires further study.

Embryo maturation, germination, and conversion are interdependent processes —only morphologically and physiologically mature embryos can successfully germinate and convert. GA_3 plays a vital role in somatic embryo development, radicle growth, and complete plantlet formation (Ammirato, 1983). Low sucrose concentrations maintain embryogenic callus with high regeneration capacity and normal seedling percentages (Abdoulaye, 2006). Jiang et al. (2013) demonstrated that GA_3 supplementation promoted somatic embryo conversion in peanut, while Zhao (2009) reported that GA_3 and low sucrose enhanced mature embryo germination. Our orthogonal experiment identified IBA 0.3 mg · L^{-1} + GA_3 0.4 mg · L^{-1} + sucrose 10 g · L^{-1} as the optimal germination combination (89.17% rate), consistent with these findings. Activated carbon benefits embryo maturation (Wang, 2016), and our results confirm its effectiveness in improving 'Ningqi 8' regeneration rates and root development. The optimal regeneration combination (IBA 0.1 mg · L^{-1} + KT 0.4 mg · L^{-1} + activated carbon 1 g · L^{-1}) aligns with Wang et al. (2004), who reported that activated carbon promoted soybean somatic embryo conversion, root growth, and vigorous seedlings by adsorbing inhibitory substances and excess hormones, thereby optimizing hormone balance (Yuan et al., 2003).

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Note: Figure translations are in progress. See original paper for figures.