Nitrogen and phosphorus co-starvation inhibits anthocyanin synthesis in the callus of grape berry skin

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Abstract

Background: Anthocyanins are a type of natural pigment that have high potential for development and utilization in regions like food, pharmaceutical, and cosmetic industries, with nitrogen and phosphorus starvation possibly promoting their accumulation in grapes. However, it remains unclear whether such starvation impacts the grape callus, or how the co-starvation of nitrogen and phosphorus affects the biosynthesis of anthocyanins. Here, we investigated how nitrogen starvation, phosphorus starvation, and the co-starvation of these two elements affects the synthesis of anthocyanins in the callus of grape skin. We also evaluated how these parameters participate in the Abscisic acid signaling pathway.

Results: Separate starvation of nitrogen and phosphorus, as well as nitrogen and phosphorus co-starvation, inhibited callus growth, while significantly promoting the accumulation of anthocyanins. However, co-starvation did not facilitate anthocyanin biosynthesis during the later stages of callus growth. qRT-PCR analysis showed that the expression of VvUFGT and VvmybA1 was closely related to anthocyanin accumulation in the callus under nitrogen and phosphorus starvation. Besides, we also confirmed that the abscisic acid signaling pathway was involved in anthocyanin accumulation as well as callus resistance under adverse conditions.

Conclusions: This study demonstrated how nitrogen and phosphorus starvation contribute to the regulation of anthocyanin synthesis in the callus of grapes. This information provides a basis for investigating the regulatory mechanisms of anthocyanin synthesis in grapes, as well as theoretical support for the production of anthocyanins by callus culture.

Keywords: grape; callus; anthocyanin; nitrogen starvation; phosphorus starvation; co-starvation

Background

Grapes (Vitis vinifera L.) are cultivated in a variety of climates on six of the seven continents. According to the International Organisation of Vine and Wine (OIV), the world's wine-growing area in 2018 covered 7.4 kha, with global grape production of 78 mt. Thus, grapes have become the most widely grown fruit in the world [1, 2, 3].

Anthocyanins are a subgroup of the flavonoids, and are secondary metabolites [4] that contribute to various biological functions in both plants and humans. For plants, Anthocyanins attract pollinators and plant seed dispersers, in addition to protecting them from abiotic and biotic stresses [5]. In parallel, anthocyanins exhibit multiple benefits to human health such as anti-inflammatory, anti-cancer, alleviate oxidative stress, prevent cardiovascular disease, control obesity and alleviate diabetes [6]. As natural pigments, anthocyanins are already used in the food, pharmaceutical, and cosmetic industries [7]. Thus, anthocyanins have long attracted the interest of researchers and, more recently, have become a popular research focus. Both the skins and pomace extract of grapes are rich in polyphenols, especially anthocyanins [8]; thus, the prospects for the development and application of grape anthocyanins are considerable.

Both nitrogen and phosphorus are macronutrients that are essential for plant growth and development [9, 10]. Many researchers have reported that nitrogen and phosphorus starvation promote the biosynthesis of anthocyanin in several crop and model species. For example, anthocyanin was observed to accumulate in the callus cultures of 'Fuji' apple when 10 mM potassium phosphate was applied, but then decreased with an increasing concentration of potassium phosphate. Accumulation was also observed at 10 mM nitrogen but decreased at 50 mM nitrogen [11]. In addition, when a novel P-deficiency mutant of Arabidopsis thaliana was cultured on medium with no added phosphorus, the growth of the pho3 mutant declined, and the anthocyanin content was enhanced 100 fold as compared to the levels in the wild-type [12]. Yin et al. [13] reported that, when compared to cells cultured in Murashige and Skoog (MS) medium containing 1.25 mM phosphate, cells cultured without phosphate accumulated significantly higher levels of anthocyanins. Such responses to nitrogen and phosphorus stress could also be applied to stimulate anthocyanin biosynthesis in grape production. Indeed, Jezek et al. [14] reported that an appropriate reduction in nitrogen or phosphorus fertilization could be used to enrich the anthocyanin concentration of grape berries and, consequently, to improve the quality of the resulting wine. Lillo et al. [15] reported that both late flavonoid pathway genes (DFR and ANS) and common MYB transcription factor genes (PAP1 and PAP2) are induced by deficiencies in the availability of certain nutrients, including nitrogen and phosphorus, thus enhancing the synthesis of flavonoids. Therefore, changes to gene expression might contribute to the accumulation of anthocyanin.

Anthocyanins biosynthesis is regulated by a variety of internal and external factors, including genetic regulation, plant hormone levels, non-hormone chemicals, and cultivation conditions [16]. In addition, the long phenological stages [17] further complicate the study and development of grape anthocyanins. Therefore, a feasible method for analyzing the formation and regulatory mechanisms of secondary metabolites, such as anthocyanins, at the cellular level is required, and could potentially be provided by culturing the callus.

The plant callus is a mass of plant cells that originate from almost any part of the plant. The callus can be maintained indefinitely in vitro by periodically passing cells and can be differentiated into complete plantlets under appropriate conditions. Callus cultures allow explants to be grown under artificially controlled conditions, where uncontrollable external conditions (such as seasonal, climate, and geographical factors) are absent. This technology has been used to produce agricultural plants, horticultural plants, therapeutic antibodies, and secondary metabolites. It facilitates the production of consistently high-quality secondary metabolites under controlled conditions. Thus, now, it is necessary to develop and market more callus culture-based products [18]. The callus has become an important experimental material for biotechnology applications, such as the use of callus culture in models for studying interactions between plants and environmental factors. Thus, the present study used callus culture to investigate the regulatory mechanisms of anthocyanin accumulation at the cellular level to elucidate how environmental stressors affect the biosynthesis of anthocyanins in vivo.

Recent studies have reported that nitrogen and phosphorus starvation effectively promote the synthesis of anthocyanins in several crop and model plant species, including grapes. However, the mechanisms

underlying this synthesis remain unclear. Fortunately, callus culture provides a unique opportunity to study this phenomenon under controlled experimental conditions. However, it remains unclear whether nitrogen and phosphorus starvation also induce the accumulation of anthocyanins in grape callus, or whether nitrogen and phosphorus co-starvation is required to enhance this process.

This study investigated the effects of nitrogen starvation, phosphorus starvation, and their co-starvation on the accumulation of anthocyanin and the expression of abscisic acid (ABA) signaling pathway genes in the callus derived from the skin of Cabernet Sauvignon (Vitis vinifera). Through this study, we expect to establish a theoretical foundation for investigating the regulatory mechanisms underlying anthocyanin synthesis in grapes and for the production of anthocyanins using callus cultures.

Results

Callus Growth

The size and color of the callus cultures (Fig. 1) were consistent with callus growth and anthocyanin content. Different growth patterns were observed in the four treatment groups (Fig. 2). There were no significant differences among the four groups after 5 or 10 d, but significant differences were observed thereafter. Group A exhibited optimum callus growth, and the growth curve followed an S-shaped pattern. After 25 d, the fresh weight of Group A reached 10.11 g, which was 3.74 times that of the initial callus weight. Even though the effect of nitrogen starvation (Group B) on callus growth was not as significant as that observed in Group A, it was 0.53 times that of Group A after 25 days. However, the callus growth was relatively stable in Group B. The fresh weights of Groups C and D were significantly lower than those of Group A. The co-starvation treatment had the most obvious inhibiting effect, with negative growth (-0.14 and -0.13 g) occurring after 20 and 25 d, respectively.

Anthocyanin Content

The anthocyanin content of the callus was significantly affected by nitrogen and phosphorus availability (Fig. 3), which was shown by the callus color. The anthocyanin content of Groups A, B, and C increased with culture duration, whereas that of Group D first increased and then decreased. When compared to the other three treatment groups, the anthocyanin content of Group A was consistently the lowest, except after 25 d. Groups B and C exhibited noticeably enhanced levels of anthocyanin accumulation after 5 d. However, even though the anthocyanin content of both Groups B and C increased rapidly during early culture, the rate of accumulation in Group C slowed during later culture. Despite this, after 25 d, Group C had the highest anthocyanin content, which was 3.04, 1.09, and 3.9 times greater than the anthocyanin levels of Groups A, B, and D, respectively. Interestingly, the anthocyanin content of Group D was significantly higher than that of the other three treatment groups after 5 and 10 d, which were 6.97 and 3.19 times higher than those of Group A, respectively. However, the positive effect of co-starvation on

anthocyanin accumulation gradually weakened. After 25 d, the anthocyanin content of Group D was the lowest of all four treatment groups, and only 78% that of Group A.

Anthocyanin Distribution

Eleven monomeric anthocyanins were detected, identified, and quantified by HPLC-ESI-MS (High-performance liquid chromatography-electrospray ionization tandem-mass spectrometry) in the callus (Table 1). The most abundant anthocyanin was peonidin-3-o(trans-6-o-coumary)-glucoside, which reached 25.30 $mg•kg^{-1}$ in Group C, followed by malpeonidin-3-o(6-o-coumary)- glucoside, peonidin-3-o-glucoside, and cyanidin-3-o-glucoside, respectively. Among the monomeric anthocyanins that were present in Groups A, B, and C, the contents in Groups B and C were significantly higher than those in Group A. For example, the peonidin-3-o-glucoside contents of Groups B and C were 7.78 and 9.40 times more than that of Group A, respectively, confirming that nitrogen and phosphorus starvation (but not co-starvation) promote the accumulation of anthocyanins. Group C had the greatest number of detectable monomeric anthocyanins (n = 11), which was four and three more than the number detected in Groups A and B, respectively. Petunidin-3-o-glucoside and petunidin-3-o(6-o-coumary)-glucoside were only detected in Group C.

Table 1. Effect of nutrient starvation on the anthocyanin contents ($mg•kg^{-1}$) of 25-d-old grape callus.

	Group A	Group B	Group C	Group D
Anthocyanin	N^+P^+	N^-P^+	N^+P^-	N^-P^-
delphinidin-3-o-glucoside	ND	0.20 ± 0.039	0.47 ± 0.028	ND
cyanidin-3-o-glucoside	1.11 ± 0.0071c	10.72 ± 0.064a	9.47 ± 0.024b	ND
petunidin-3-o-glucoside	ND	ND	0.12 ± 0.011	ND
peonidin-3-o-glucoside	1.59 ± 0.0081c	12.37 ± 0.061b	14.94 ± 0.0097a	ND
malvidin-3-o-glucoside	ND	0.97 ± 0.0030	1.48 ± 0.047	ND
peonidin-3-o(6-o-acety)-glucoside	0.65 ± 0.060c	3.04 ± 0.088b	5.96 ± 0.0014a	ND
malpeonidin-3-o(6-o-coumary)-glucoside	1.67±0.0081c	7.68 ± 0.015b	11.37 ± 0.0070a	ND
petunidin-3-o(6-o-coumary)-glucoside	ND	ND	0.29 ± 0.035	ND
peonidin-3-o(cis-6-o-coumary)-glucoside	0.28 ± 0.20	ND	0.99 ± 0.033	ND
peonidin-3-o(trans-6-o-coumary)-glucoside	4.29 ± 0.62c	13.08 ± 0.0045b	25.30 ± 0.017a	ND
malvidin-3-o-(trans-6-o-coumary)-glucoside	1.76 ± 0.41b	2.03 ± 0.0061b	4.26 ± 0.025a	ND

Legend: Data are expressed as mean value per plate ± standard deviation (n = 6). Lowercase letters indicate significant differences between treatment groups ($p < 0.05$, Duncan's multiple). ND means "not detected". No anthocyanins were detected in group D. N^+, nitrogen; N^-, nitrogen starvation; P^+, phosphorus; P^-, phosphorus starvation.

Nitrogen and Phosphorus Levels

The nitrogen contents of Groups A and C were increased by the callus culture; however, the nitrogen content of Groups B and D (which were nitrogen-starvation treatment) declined (Fig. 4). The effects of phosphorus starvation on the total phosphorus contents of the callus were inconsistent; however, the total phosphorus levels of Groups B, C, and D were lower than those of Group A throughout most of the culture period. Thus, phosphorus starvation affected the absorption of phosphorus by the callus. Thus, both nitrogen and phosphorus starvation influence the total nitrogen and phosphorus contents of the callus.

Expression of Anthocyanin Biosynthesis Genes

The relative expression of anthocyanin synthesis and regulation-related genes was measured over 25 d. The relative expression of anthocyanin synthesis-related genes was greater in Groups B and C than in Group A throughout the culture process. *VvCHS1*, *VvCHS2*, *VvCHS3*, *VvF3H1*, and *VvF3H2* might be key genes for the accumulation of anthocyanins under nitrogen starvation, while *VvCHS1*, *VvCHS2*, *VvCHS3*, *VvF3H1*, and *VvLDOX* might be key genes for the same under phosphorus starvation (Fig. 5). In general, the expression of most genes in Group C was much higher than that in Group A during the first 20 d, but was more similar after 25 d.

The relative expression of most genes in Group D was significantly different to that in A. The changes in the expression of each gene were relatively consistent; they first increased and then decreased, with maximum gene expression occurring at 10 d. Until 20 d after inoculation, the relative expression of *VvCHS1*, *VvCHS2*, *VvCHI*, *VvF3H1*, *VvF3H2*, *VvDFR*, and *VvLDOX* in Group D was lower than that in Group A. Therefore, when compared to Group A, the co-starvation treatment actually upregulated the expression of anthocyanin biosynthesis-related genes during the first 10 d; however, the upregulation was subsequently weakened or, even, inhibited, which was coincident with the decline in anthocyanin content during the late-stage culture.

Expression of ABA Signaling Pathway Genes

Separate nitrogen and phosphorus starvation had similar effects on the expression of ABA signaling pathway genes (Fig. 6). The expression of the ABA-synthesis gene *VvNCED1* was significantly upregulated, whereas the two ABA-degrading genes, *VvHYD2* and *VvGT1,* were downregulated. Even though the expression of *VvNCED1* in Group D was upregulated throughout the experiment, *VvHYD2* was also significantly upregulated 20 d after inoculation, at which point the expression of *VvHYD2* in Group D was 14.70 times more than that in Group A. The expression of *VvGT1* started decreasing 10 d after inoculation, but remained higher than that of Group A.

Discussion

Previous studies have confirmed that nitrogen and phosphorus enhance the biosynthesis of anthocyanin in the field. For example, Soubeyrand et al. [19] reported that the total anthocyanin content of field-grown grapes cultivated under low-nitrogen conditions was significantly greater than that of grapes cultivated under high-nitrogen conditions. This phenomenon occurred because nitrogen starvation upregulates both the structural and regulatory genes involved in anthocyanin synthesis. Therefore, the appropriate reduction in nitrogen or phosphorus fertilization under culture conditions could increase anthocyanin content and, consequently, enhance fruit quality. However, it remains unclear whether nitrogen and phosphorus starvation have the same effect on grape callus. Therefore, the present study investigated how nitrogen and phosphorus starvation affect anthocyanin biosynthesis and the ABA signaling pathway

of the grape callus, along with the underlying genetic mechanisms that regulate changes in the anthocyanin content.

Isah [20] demonstrated that greater productions of secondary compounds in plants prevents free radicals from causing damage associated with nutrition-based stress. As an important secondary metabolite, anthocyanin has several functional roles in plant-environment interactions, and it hinders the progression of senescence under mineral deficiencies, such as deficiencies in nitrogen or phosphorus [21]. Liang and He [22] recently reported that nitrogen-starved Arabidopsis plants exhibit retarded growth and enhanced anthocyanin accumulation; thus, anthocyanins likely contribute to the tolerance of plants to low-nitrogen stress. In the present study, nitrogen and phosphorus starvation inhibited callus growth, but induced anthocyanin accumulation. In addition, separate nitrogen and phosphorus starvation significantly upregulated the expression of almost all the tested structural and regulatory genes involved in anthocyanin synthesis, supporting existing research [13, 22, 23]. Therefore, the results of the present study support that primary metabolism (mainly callus growth) is dominant when nitrogen and phosphorus are readily available. Thus, sufficient nitrogen and phosphorus levels are vital for callus growth. The accumulation of anthocyanins showed that secondary metabolite systems are stimulated by nitrogen or phosphorus starvation, and that anthocyanin biosynthesis might serve as a defense mechanism against environmental stress. Combined with the growth and accumulation of anthocyanins of the callus, we showed that the nitrogen starvation treatment represents an appropriate model to produce anthocyanin, because it promoted steady callus growth and anthocyanin accumulation throughout the culture period. Up to 11 anthocyanins were detected in the callus in our study, with previous studies detecting 19 anthocyanins in the skin of ‘Cabernet Sauvignon’ berries [24]. Thus, the biosynthesis of anthocyanins appears to be somewhat restricted in the callus.

In contrast to what was observed during separate starvation, nitrogen and phosphorus co-starvation initially enhanced anthocyanin accumulation, but later inhibited it. Indeed, the relative expression of genes under this treatment also increased initially, and then decreased after peaking at 10 d after inoculation. Peng et al. [25] showed that, in contrast to wild-type plants, the Arabidopsis nla (nitrogen limitation adaptation) mutant could not control nitrogen limitation-induced anthocyanin synthesis; consequently, it exhibits early senescence. However, under co-starvation conditions, the mutant accumulated anthocyanins, allowing it to adapt to adverse conditions. Therefore, interactions between nitrogen and phosphorus might affect how plants respond to adversity; in fact, such interactions might have driven the pattern of anthocyanin accumulation detected under co-starvation conditions in the present study. Finally, due to the absence of nitrogen and phosphorus in the co-starvation treatment, the original nutritional elements of the media could not support callus growth. Specifically, the physiological activity of the callus, including its resistance to nutrient starvation, decreased with culture duration, thus limiting anthocyanin biosynthesis. In addition, at least until 25 d after inoculation, the callus appeared to have been inactivated, making it difficult to extract RNA for analysis. Overall, additional research is needed to elucidate the interactions between co-occurring nitrogen and phosphorus starvation.

In the present study, changes of anthocyanin content were shown both in callus color and the level of anthocyanin biosynthesis-related genes transcripts. The final step in the flavonoid pathway is the transformation of anthocyanidins to water-soluble anthocyanins, which are more stable and deeper in color. This process is under the regulation of UFGT, which is under the control of VvMYBA1. Accordingly, the VvMYBA1 factor is considered a major gene that determines anthocyanin synthesis and the color of grape skin [26, 27, 28]. In the present study, the relative VvUFGT and VvmybA1 expression of the three starvation treatments was significantly upregulated during the early stages of culture, but was relatively unaffected (i.e., similar to the control group) during later stages. Thus, nitrogen and phosphorus starvation likely promote the pre-expression of these two genes. Therefore, the early expression of VvUFGT and VvmybA1 might be associated with anthocyanin accumulation under nitrogen and phosphorus starvation in the callus.

Many researchers have investigated the regulatory effects of nitrogen and phosphorus starvation on anthocyanin accumulation. For example, Jiang et al. [29] reported that phosphorus starvation reduces the level of bioactive gibberellin (GA) in Arabidopsis, thus promoting DELLA accumulation (core components of the GA-signaling pathway). As a consequence, several adaptively significant plant phosphorous-starvation responses are modulated, including the inhibition of growth, accumulation of anthocyanins, and elongation of root hairs. Using both molecular and genetic approaches, Lei et al. [30] demonstrated that ethylene signaling upregulates phosphorus starvation-induced genes and acid phosphatase, while negatively regulating the accumulation of anthocyanin. Thus, plant hormones are clearly related to anthocyanin production under nutrient-starvation conditions. The current study investigated the relationship between Abscisic acid (ABA) and anthocyanin production under nitrogen and phosphorus starvation. ABA is a key component of plant growth and development associated with the environment. As a sesquiterpene plant hormone that regulates a variety of plant processes, ABA helps plants to adapt to abiotic and biotic stressors [31], thus contributing to the responses of plants to adverse environmental conditions. Several studies reported that exogenous ABA application promotes the synthesis of anthocyanins in grapes [32, 33, 34]. In particular, Jia et al. [34] reported that VvMYBA2 and VvUFGT are significantly upregulated in ABA-treated berries, contributing to elevated anthocyanin content. The present study evaluated the relative expression levels of five genes related to the abscisic acid signaling pathway, based on Ferrero et al. [35]. Our results showed that separate nitrogen and phosphorus starvation upregulated the key gene of the ABA-synthesis pathway (VvNCED1), and downregulated the ABA-degradation genes (VvHYD2 and VvGT1). Furthermore, the levels of anthocyanins that accumulated under these two treatments were significantly elevated. The expression of VvNCED1 was partly upregulated, whereas the expression of VvHYD2 significantly increased during late stages of culture. Compared to Group A, the expression of VvGT1 was consistently higher under nitrogen and phosphorus co-starvation.

Therefore, the callus might synthesize ABA by upregulating ABA-synthesis genes and downregulating ABA-degrading genes when exposed to separate nitrogen and phosphorus starvation. This phenomenon might promote the biosynthesis of anthocyanins and improve the ability of the callus to adjust to nutrient-deficient environments. The downregulation of ABA-synthesis genes and upregulation of ABA-

degrading genes during the late stage of co-starvation treatment might be related to the downregulation of anthocyanin biosynthetic genes. Consequently, this phenomenon might reduce anthocyanin content in the callus.

Conclusions

Grape callus culture is an appropriate system for investigating how nitrogen and phosphorus starvation affect anthocyanin synthesis in grapes. This study demonstrated that separate nitrogen and phosphorus starvation inhibited callus growth but promoted anthocyanin biosynthesis. This phenomenon was attributed to changes in the expression of certain genes (e.g., VvUFGT and VvMYBA1), which are related to ABA-mediated anthocyanin biosynthesis. The upregulation and downregulation of ABA-degrading and synthetic genes, respectively, might inhibit the accumulation of anthocyanin accumulation and lower the tolerance of the callus to stress caused by nitrogen and phosphorus co-starvation. This phenomenon ultimately causes the inactivation of the callus.

Methods

Callus Culture Methods

The callus of grape berry skin used in the present study were kindly provided by Prof. Jicheng Zhan from the College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China. The callus was induced from the skins of *Vitis vinifera* cv. 'Cabernet Sauvignon' grapes [36]. It was sub-cultured on complete (A: N^+P^+), nitrogen-deficient (B: N^-P^+), phosphorus-deficient (C: N^+P^-), and co-deficient (D: N^-P^-) MS medium. All media were supplemented with 3% sucrose, 0.3% phytagel, 0.1% inositol, 0.03% KNO_3, 1.126 mg•L^{-1} 6-BA (6-benzylaminopurine), and 0.55 mg•L^{-1} NOA (naphthoxyacetic acid). The pH of the media was adjusted to 5.8–5.9 before autoclaving for 20 min at 121 °C (Shanghai Bo Xun Medical Biological Instrument Co., Ltd., Shanghai, China). In total, 2.7 g callus was inoculated in each medium. It was then transferred to a tissue culture room, where it was maintained at 25 ± 1 °C under a 16 h light photoperiod (2000–2400 Lx) and relative humidity of 60–70%.

Growth Measurement

Six plates of callus were harvested from the four groups on days 0, 5, 10, 15, 20, and 25 after inoculation. During each sampling event, the fresh weight of the callus in each plate was first measured. The callus was then quickly frozen using liquid nitrogen, and stored at −80 °C until analysis.

Anthocyanin Extraction and Measurement

The callus cultures were harvested on days 0, 5, 10, 15, 20, and 25 d after inoculation. The callus cultures were then ground to powder using liquid nitrogen. Each resulting powder sample (0.5 g) was dissolved in 10 mL 60% methanol extract, which contained 0.1% HCl. The supernatant was collected after ultrasound extraction for 30 min at 40 W (Kunshan Ultrasonic Instruments CO., Ltd. Kunshan, Jiangsu, China). It was then centrifuged for 10 min at 8000 ×g. The remaining residue was subjected to another 10 mL extract for two additional rounds of extraction [37]. The anthocyanin content ($mg \bullet g^{-1}$) of the pooled extract solutions was estimated using the pH differential method described by Lee et al. [38].

Anthocyanin Identification

Twenty-five-day-old callus samples were ground to powder using liquid nitrogen under dark conditions. They were then pre-cooled to −40 °C for 30 min, and freeze-dried using a lyophilizer (Biocoll, Beijing, China). Portions of each dry powder (0.25 g) were transferred to separate 50 mL centrifuge tubes and mixed with 5 mL of a 98:2 (v:v) mixture of methanol and formic acid. The resulting supernatant was collected after the samples were subject to ultrasound extraction for 10 min (20 °C, 100 W) and then placed on a shaker (Zhicheng, Shanghai, China) for 30 min (25 °C, 130 rpm), followed by centrifugation for 10 min at 8000 ×g. For each sample, the precipitate was re-extracted with another 5 mL extract for three additional rounds of extraction. Finally, the pooled extraction supernatants were subjected to rotary evaporation at 30 °C (Shanghai Shensheng Technology Co., Ltd. Shanghai, China) until they were dry. The final sediment was diluted to 5 mL with a mixture containing mobile phase A (water:formic acid:acetonitrile = 92:2:6, v:v:v), and one volume of mobile phase B (water:formic acid:acetonitrile = 44:2:54, v:v:v). The mixture was stored at −80 °C for subsequent analysis [39].

Aliquots (30 μL) of each diluted extract were analyzed three times by HPLC-ESI-MS using an Agilent 1100 series LC-MSD trap VL (Agilent Corporation, Santa Clara, CA, USA), which was equipped with a de-gasser (G1379A), quaternary pump (G1311A), ALS autosampler (G1313A), photodiode array detector (G1315B), and reversed-phase column (Kromasil C18, 250 × 4.6 mm i.d., 5 μm). The mobile phase contained: phase A, water:formic acid:acetonitrile (92:2:6, v:v:v); phase B, water:formic acid:acetonitrile (44:2:54, v:v:v). The column was maintained at 50 °C, and the gradient elution had the following proportions (v/v) of solvent B: 0–1 min, 10%; 1–18 min, 10–25%; 18–20 min, 25%; 20–30 min, 25–40%; 30–35 min, 40–70%; 35–40 min, 70–100%; 40–45 min, and 100–10%, with a flow rate of 1.0 $mL \bullet min^{-1}$. Quantification was achieved by peak area measurement at 525 nm. The MS (mass spectrometry) conditions were: electrospray ionization interface, positive ion model, nebulizer, 35 psi, 10 $mL.min^{-1}$ dry gas (325 °C) flow rate, and scans between 100–1000 $m \bullet z^{-1}$ [40].

Gene Expression Analysis

Total RNA was isolated from callus samples collected on day 0, 5, 10, 15, 20, and 25 after inoculation using the Universal Plant Total RNA Extraction Kit (Bio Keke, Beijing, China). Then, 50 μL of the RNA system was obtained and preserved at −80 °C. A quantity of 500 ng RNA was used to synthesize cDNA through HiScript II Q Select RT SuperMix for qPCR Kit (Vazyme, Nanjing, China). The cDNA products were diluted 20 times and stored at −20 °C. Finally, quantitative real-time polymerase chain reaction (qRT-PCR) analyses were conducted using ChamQ SYBR qPCR Master Mix Kit (Vazyme, Nanjing, China), with 2 μL template cDNA and 0.4 μL of each gene-specific primer (Table 2). The investigated anthocyanin biosynthesis genes included nine structural genes (*VvCHS1*, *VvCHS2*, *VvCHS3*, *VvCHI*, *VvF3H1*, *VvF3H2*, *VvDFR*, *VvLDOX*, and *VvUFGT*) and a regulatory gene (*VvmybA1*). Abscisic acid signaling pathway genes included *VvNCED1* (a key gene in ABA synthesis), *VvHYD2* (involved in ABA hydroxylation), *VvGT1* (involved in ABA conjugation), *VvBG1* (involved in ABA de-conjugation), and *VvABCG40* (a plant transporter involved in ABA absorbance). *VvATIN* was used as the internal standard gene. Three biological and technical replicates were performed for each template cDNA. After qRT-PCR, CT values were calculated, the relative expression levels of the target genes were determined using the $2^{-\Delta\Delta CT}$ method, as described by Livak and Schmittgen [41].

Table 2. Quantitative RT-PCR primers.

	Sequence of forward primer (5'-3')	Sequence of reverse primer (5'-3')
VvCHS1	TTCCGGATCACCAACAGTGAG	CACCATGTCTTGACGGGCAT
VvCHS2	GAGAACCCCAACGTCTGTGC	TAGTCAGCACCGGGCATGT
VvCHS3	TCGCATCACAAATAGCGAACAC	GCTGCCTCTTTGCCTAGCTT
VvCHI	GTCAGTCACCGCAGTTCAGG	CCACAGTCTTGCCCTTCCAC
VvF3H1	GCTCGGGAATTCTTCGCTCT	TCGAGTAGTCCCTGGTTCGT
VvF3H2	GCTCGGACTCAAACGGCATA	GCAGTCGGAGTTCACCACAG
VvDFR	GGCTTTCTAGCGAGAGCGTAG	GAGACCACCTTGGGCCATTC
VvLDOX	CCTGAGGACAAGCGCGATA	CCAATCCCAACCCAAGCGAT
VvUFGT	TGCAGGGCCTAACTCACTCT	CGATGGAGCATGCGTGAGAA
VvMybA1	TAGTCACCACTTCAAAAAGG	GAATGTGTTTGGGGTTTATC
VvABCG40	CTGTTGAGGCTCAGGAATCGT	CAAGGCATCCTCGATTTTATTGAAC
VvBG1	GCAAACAACTTGACTGGGATGC	CCTTCTCATCCCACGAGGTACT
VvGT1	GGTTGCTTCCCTCGATGTGG	CATCATCTTCGGGCGCTTGT
VvHYD2	TATTCAGTATGGCCCTTTTGCT	TTGATTGGTGGCACTGAGAG
VvNCED1	TCCACGGCACCTTCATAAGC	GGAGATTCCGGGGACGCATA
VvATIN	CTTGCATCCCTCAGCACCTT	TCCTGTGGACAATGGATGGA

Nitrogen and Phosphorus Measurement

The callus samples collected on days 0, 5, 10, 15, 20, and 25 after inoculation were ground to powder using liquid nitrogen. They were then sent to the Food Quality Inspection Center of the Ministry of Agriculture and Rural Areas of Yangling, where the total nitrogen and phosphorus levels of the callus samples were determined using a nitrogen analyzer and colorimetry, respectively.

Statistical Analysis

All the experimental data were collated using EXCEL.2010 (Microsoft, Redmond, Washington, USA), and were analyzed using SPSS.21 (SPSS Inc., Chicago, USA). The significance of differences between group means was assessed at the $p < 0.05$ level (Duncan's multiple).

Abbreviations

OIV: Organisation of Vine and Wine; MS: Murashige and Skoog medium; *nla*: Nitrogen limitation adaptation; GA: Gibberellin; ABA: Abscisic acid; HPLC-ESI-MS: High performance liquid chromatography-electrospray ionization-mass spectrometry; 6-BA: 6-benzylaminopurine; NOA: Naphthoxyacetic acid.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests in this section.

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Authors' contributions

JFM and TFX designed the research and proposed the research process. HZZ, HW, SHG, MXF, and XQJ performed the experiments. YLF and ZWZ formulated the experimental platform. HZZ analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Figures

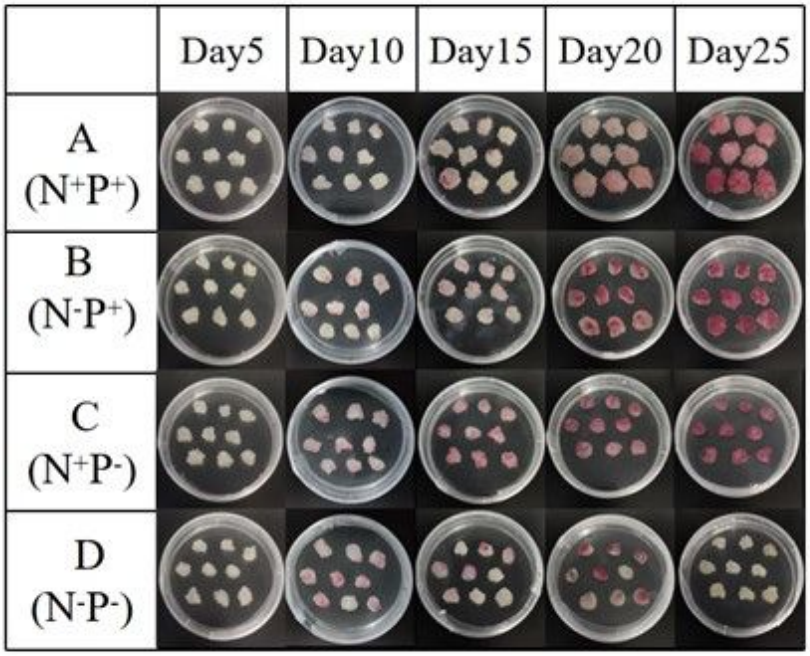

Fig. 1

Figure 1

Effect of nutrient starvation on the phenotypes of the skin callus of grapes. N+, nitrogen; N-, nitrogen starvation; P+, phosphorus; P-, phosphorus starvation.

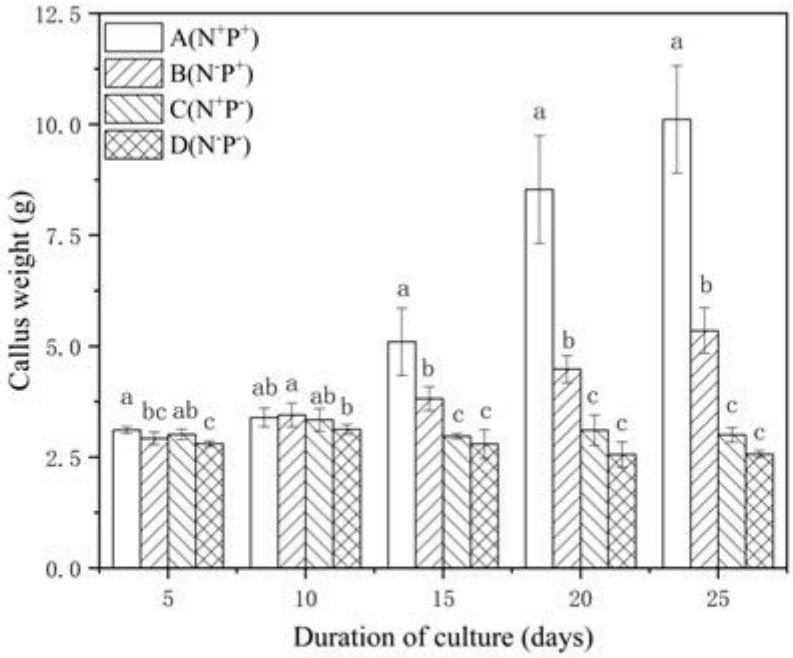

Fig. 2

Figure 2

Effect of nutrient starvation on callus growth. Data are expressed as mean value per plate ± standard deviation (n = 6). Lowercase letters indicate significant differences between treatment groups ($p < 0.05$, Duncan's multiple). N+, nitrogen; N-, nitrogen starvation; P+, phosphorus; P-, phosphorus starvation.

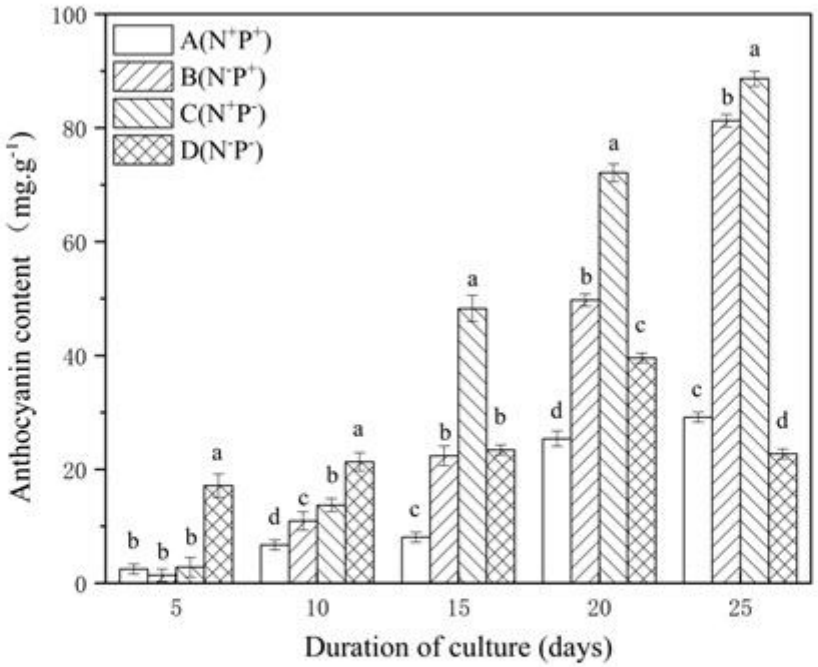

Fig. 3

Figure 3

Effect of nutrient starvation on callus anthocyanin content. Anthocyanin levels were calculated using the pH differential method. Data are expressed as mean value per plate ± standard deviation (n = 6). Lowercase letters indicate significant differences between treatment groups ($p < 0.05$, Duncan's multiple). N+, nitrogen; N-, nitrogen starvation; P+, phosphorus; P-, phosphorus starvation.

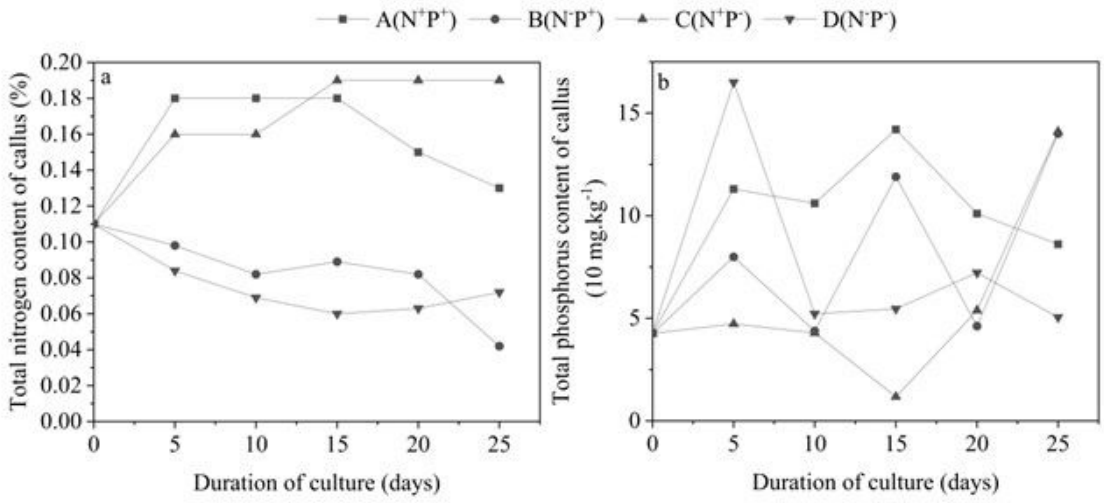

Fig. 4

Figure 4

Effect of nutrient starvation on the nutrient content of the callus. (a) total nitrogen content; (b) total phosphorus content. Values indicate means (n = 3). Statistical analysis indicated that there were no significant differences among replicates. N+, nitrogen; N-, nitrogen starvation; P+, phosphorus; P-, phosphorus starvation.

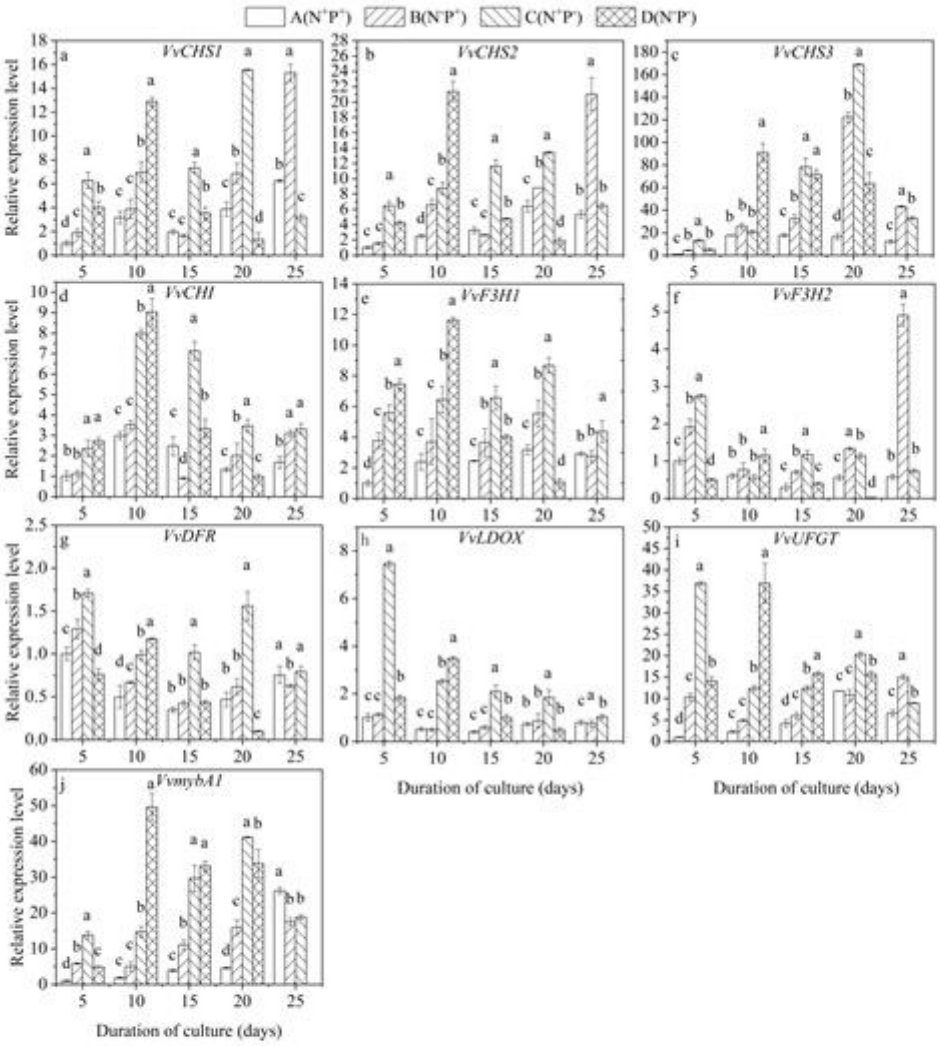

Fig. 5

Figure 5

Effect of nutrient starvation on the relative expression levels of anthocyanin biosynthesis-related genes; (a-i) structural genes in the anthocyanin biosynthesis pathway; (j) regulator gene in the anthocyanin biosynthesis pathway. Data are expressed as mean value per plate ± standard deviation (n = 6). N+, nitrogen; N-, nitrogen starvation; P+, phosphorus; P-, phosphorus starvation.

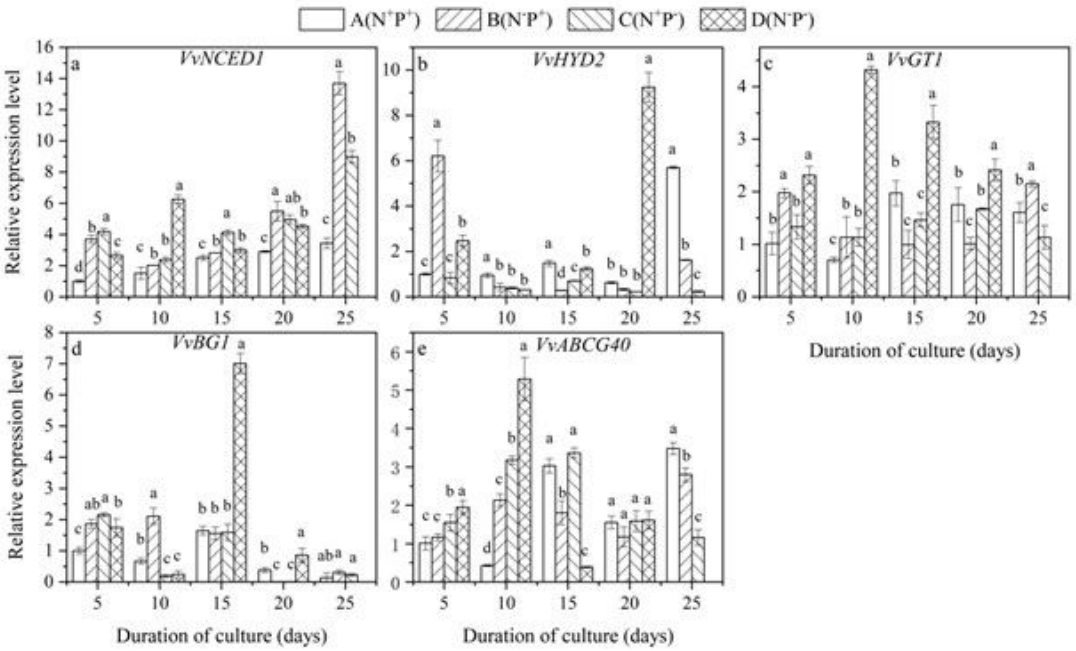

Fig. 6

Figure 6

Effect of nutrient starvation on the relative expression levels of ABA signaling pathway-related genes. Data are expressed as mean value per plate ± standard deviation (n = 6). N+, nitrogen; N-, nitrogen starvation; P+, phosphorus; P-, phosphorus starvation.