

Establishment of hairy roots culture of *Perilla frutescens* L. and production of phenolic acids

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Abstract

Perilla frutescens L. is a kind of phenolic acids rich plant. Rosmarinic acid (RA) is the main phenolic acid component in perilla. It has various pharmacological activities such as anti-allergic, anti-inflammatory, and antioxidant. In this study, the hairy roots culture of *Perilla* was established from leaves explants using *Agrobacterium rhizogenes* C58C1, A4, R1000, and the content of phenolic acids was also determined. The highest RA content in the five obtained fast-growing lines reached 19.08mg/g (DW). The effects of salicylic acid (SA) and methyl jasmonate (MeJA) treatment on phenolic acids (Protocatechuic aldehyde, caffeic acid, ferulic acid, RA, and ethyl caffeic acid) accumulation in hairy roots were also investigated. SA can promote phenolic acid accumulation; the content of RA reached 20.92 mg/g (DW) on day nine after treatment. MeJA can upregulate the RA content to 17.36mg/g (DW) on day three, and then the RA content declined to 4.27mg/g (DW) on day 9. These results showed the high potential of perilla hairy roots to produce phenolic acids. It would be an efficient method for the large-scale production of RA in *P. frutescens*.

1. Introduction

Perilla frutescens L. is an annual herb belonging to the *Lamiaceae* family, originating from East Asia and recognized as a traditional Chinese medicinal and edible plant. The usage of *Perilla* in traditional medicine involves treating ailments like coughs, headaches, colds, asthma, nasal congestion, chest tightness, and constipation. Modern pharmacological research has noted that *Perilla* has many pharmacological activities ranging from anti-inflammatory (Lee et al., 2014), anti-allergic (Oh et al., 2011), antioxidant (Hong et al., 2011), anti-cancer (Kwak and Ju, 2015), antibacterial (Kim et al., 2011), anti-depressant (Kim et al., 2011) and liver-protective effects (Zhang et al., 2018), among others. Studies related to phytochemical reports have identified that phenolic acids are the primary active ingredients found in perilla; RA is the leading phenolic acid present in *Perilla*. RA is generally available in *Lamiaceae* plants, and it can be easily detected in the leaves, seeds, and stems - the principal medicinal parts of *Perilla* prescribed by the Chinese Pharmacopoeia.

Agrobacterium rhizogenes is a soil bacterium that belongs to the group of gram-negative bacteria and has a broad range of hosts. Its root-inducing (Ri) plasmid has a complete T-DNA, and it carries the *rol* oncogenes (*rolA*, *B*, *C*, and *D*) (Chilton et al., 1982). The basic principle behind hairy root culture involves infecting the recipient plant cells with *A. rhizogenes* to integrate the T-DNA in the Ri plasmid into the genomic DNA of the recipient cells, and *A. rhizogenes* infects the injured parts of the plant to induce the growth of hairy roots (Chandra and Chandra, 2011). Hairy roots possess different characteristics from the original plant, such as hormone-free nature, rapid growth, genetic stability, and others (Wu et al., 2007). Many researchers have shown that the production of various secondary metabolites using hairy roots culture is feasible, and these compounds are present at significantly higher levels than in the original plants. Due to its ability to produce valuable natural products on a large scale, hairy roots culture holds promise for industrial applications.

Hairy root cultures have been successfully established for several *Lamiaceae* plants, such as *Salvia miltiorrhiza* (Zhang et al., 2012) and *Salvia bulleyana* (Wojciechowska et al., 2020). However, the establishment of a hairy root culture system for *Perilla frutescens* has not been reported in the literature. In this study, an efficient hairy root culture system for *P. frutescens* was established using *A. rhizogenes* C58C1. In many plant species, the production of secondary metabolites can be improved through the use of elicitors. Methyl jasmonate (MeJA) and salicylic acid (SA) are commonly used elicitors to induce the production of secondary metabolites (Sharifzadeh Naeini et al., 2021). Previous studies have shown that MeJA can promote tanshinone production in *S. miltiorrhiza* cell cultures (Zhao et al., 2010) and increase phenolic acid content in *S. miltiorrhiza* hairy root cultures (Xiao et al., 2009). Similarly, SA and MeJA were found to stimulate tropane alkaloid content in transgenic *Atropa baetica* (El Jaber-Vazdekis et al., 2008) and significantly enhance phenolic acid content in *S. miltiorrhiza* and *Lithospermum erythrorhizon* suspension cell cultures (Dong et al., 2010; Mizukami et al., 1993). Therefore, in this study, MeJA and SA were used to evaluate their effectiveness in inducing the production of secondary metabolites in *P. frutescens* hairy roots.

The current study reports the successful establishment of *Perilla frutescens* hairy roots using the *A. rhizogenes* C58C1 induction process for the first time. Five fast-growing hairy root lines were selected, and the accumulation of phenolic acids in these hairy roots was determined. The study also investigated the effects of MeJA and SA on the phenolic acid content in *P. frutescens* hairy roots. The results of this study provide a new and effective platform for research on phenolic acid production in *P. frutescens*, which could be useful in the development of new natural products with potential therapeutic applications.

2. Materials and Methods

2.1. Plant material and Preparation of *Agrobacterium rhizogenes*

The seeds of *Perilla frutescens* were obtained from the Medicinal Botanical Garden of Jilin Agricultural University. The fully matured seeds were sterilized using Mercury dichloride (HgCl₂) for 12–15 minutes and then washed with 75% ethanol for 30 seconds, followed by several washes with sterile distilled water. The sterilized seeds were then cultured on solidified MS medium containing 0.1% agar and 0.3% sucrose according to the protocol described by Murashige and Skoog in 1962 (Murashige and Skoog, 1962). The cultures were incubated at a temperature of 25°C under a 12/6h(light/dark) photoperiod in a growth incubator..

For the induction of hairy roots, three *A. rhizogenes* strains - C58C1, A4, and R1000 - were streaked on YEB liquid medium supplemented with Rifampicin (Rif)(50mg/ml) and incubated at 28°C. Single colonies were picked and sub-cultured in YEB liquid medium with Rifampicin, following which they were shaken overnight at 28°C. Finally, the density of the *A. rhizogenes* cells was adjusted to an OD_{600 nm} value of 0.6 spectrophotometrically to facilitate effective hairy root induction..

2.2. Transformation and cultures of *P. frutescens* explants

In this study, 7-week-old sterile leaves of *P. frutescens* aseptic seedlings were used as explant materials for hairy root induction. The leaves were cut into 1.0×1.0 cm pieces, and then placed in liquid 1/2MS medium containing *A. rhizogenes* strains for 15 minutes. Co-cultivation was carried out at a temperature of 28°C in a constant-temperature shaker (120 rpm) under dark conditions. Following co-cultivation, the explants were dried using sterile tissue paper and then transferred onto MS solid medium for three days under dark conditions. The leaves were washed several times with sterile distilled water, dried on sterile filter paper, and finally, placed on MS solid medium supplemented with 500mg/L Cefotaxime (Cef). The cultures were incubated at a temperature of 25°C for seven days to remove bacteria. After this, the concentration of Cef was gradually reduced each week (250, 100, 50, 0 mg/L) until the bacteria were removed from the hairy roots, which formed at the wounded sites of the explants about one week after induction.

Once the hairy roots reached a length of 5 cm, they were individually transferred to MS solid medium without Cef and cultured until their fresh weight reached approximately 0.5g. These hairy lines were then cultured in 100 mL Erlenmeyer flasks containing liquid 1/2MS medium and maintained on a rotary shaker (120 rpm) at a temperature of 25°C in the dark. During subculturing, five fast-growing lines were selected for further experiments and labeled P1 to P5.

2.3. Confirmation of transformation

The *rol* gene is present in the T-DNA region of the Ri plasmid of *A. rhizogenes*, which is usually used to identify hairy roots. By designing specific primers for *rolB* and *rolC* genes, the DNA of five hairy root clones was extracted for PCR amplified. The specific amplification primers were listed as follows: *rolB* (423bp) (Forward: GCTCTTGCAGTGCTAGATTT; Reverse: GAAGGTGCAAGCTACCTCTC); *rolC* (626bp) (Forward: CTCCTGACATCAAACGTC; Reverse: TGCTTCGAGTTATGGGTACA). The plasmid DNA of *A. rhizogenes* C58C1 was used as a positive control, and the roots of *P. frutescens* sterile seedling DNA were used as negative controls. The amplified PCR reaction products were run on 1% agarose gel (GelStain staining) electrophoresis, and the 2k DNA marker was used as a reference.

2.4 Preparation and application of MeJA and SA

Methyl jasmonate (MeJA) and Salicylic acid (SA) were used as the elicitor for hairy root elicitation. The elicitors were dissolved in ethanol and filtered by 0.22 µm filters for sterilization, respectively. After the hairy roots were cultured for 20 days, adding the elicitors into the 1/2MS liquid medium to the final concentration of 100µM. The hairy roots were harvested on days 0, 3, 6, and 9 after treatment and then dried in an oven at 50°C until the dry weight constantly.

2.5 Phytochemical analysis

2.5.1 Extraction procedure

The 200 mg powder samples of fast-growing perilla hairy roots lines(P1-P5) and the hairy roots of treatment with elicitor at different periods (0, 3, 6, 9 d) were collect and weighed accurately. Then put hairy roots powder soaked in 5ml of 70% methanol solution, treated by ultrasound for one hour. The supernatant was filtered through a 0.22 µm organic membrane and analyzed by an HPLC system.

2.5.2 Quantitative analysis

In this study, phenolic acids content was measured using High-Performance Liquid Chromatography (HPLC), HPLC (Waters USA) analysis system was equipped with e2695 system separations module and 2489 UV / Vis detector (Waters, USA), the column was Cosmosil Cholesterol packed C18 column (4.6×250 mm). The column temperature was 35°C, detection wavelength was 330 nm. The injection volume was 20 µL. The mobile phase is methanol (A)-0.2% phosphoric acid water (B). The gradient elution system was used: 0 15 min, 25% A; 15 25 min, 25% 50% A; 25 28 min, 50% A; 28 29 min, 50% 25%A; 29 34 min, 25%A. The flow rate was 1 ml/min.

2.6 Statistical analysis

All the experiments were performed in triplicate and the data presented are the mean values of three determinations. The data were calculated as mean ± standard error (SD).

3. Results

3.1 Establishment and cultures of hairy roots

3.1.1 Effect of different strains on the induction rate of hairy roots

In this study, three different strains of *A. rhizogenes*, namely R1000, A4, and C58C1, were used for the induction of hairy roots in *P. frutescens*. The effectiveness of these strains in inducing hairy root formation was evaluated, and the results showed that there were significant differences in the induction rate of hairy roots among the three strains.

After 15 days of sterilization and cultivation, the number of hairy roots produced by the perilla leaves varied among the different strains, as shown in Fig. 1A. The results indicated that the C58C1 strain had the highest number of hairy roots. During the co-cultivation stage, perilla explants infected with the C58C1 strain had hairy roots growing around the fifth day, with an induction rate of 97.7%. In contrast, perilla explants infected with the R1000 and A4 strains had hairy roots growing around the tenth and fifteenth day, respectively, with an induction rate of only 63.3% and 28.9% respectively, as seen in Fig. 1B. These findings suggest that the C58C1 strain is more effective than the other two strains in inducing hairy root formation in *P. frutescens*.

3.1.2 Effects of different infecting times and co-culture times on the induction rate of hairy roots

In this study, the infecting time and co-culture time were also evaluated to determine their effect on the induction rate of hairy roots in *P. frutescens*. (Fig. 2A, Fig. 2B) The results revealed that these factors played a critical role in influencing the success rate of hairy root induction. Regarding infecting time, the C58C1 bacterial solution with an OD value of 0.6-1 was used to infect the leaves of sterile *P. frutescens* seedlings for different durations. The results showed that the optimal infecting time was 15 minutes, where the induction rate of hairy roots reached 96.6%, as seen in Fig. 2C. Regarding co-culture time, it was observed that both short and long periods had negative effects on the induction rate of hairy roots. If the co-culture time is too short, there may be insufficient transfer of *Agrobacterium* into the explants, leading to low induction rates. On the other hand, if the co-culture time is too long, it can result in incomplete removal of the bacterial solution, causing browning of leaves, loss of growth vitality, reduced induction efficiency, or even cell death. From the results, it was found that 2–3 days is the optimal co-culture time for inducing hairy roots in *P. frutescens*. This duration provided adequate time for *Agrobacterium* transfer into the explants without causing significant harm to the explants. The results also indicated that on the fourth and fifth day of co-culture, the bacterial solution on the plate could lead to browning of the leaves, as seen in Fig. 2D.

The induction of hairy roots in *P. frutescens* was visually monitored and documented (as shown in Fig. 3). After infecting the leaves explants with *A. rhizogenes* C58C1, hairy roots were observed one week later on the wounded petiole, as seen in Fig. 3A and 3B. Subsequently, many hairy roots appeared after the second or third week of incubation, as shown in Fig. 3C. When the hairy root reached approximately 5 cm in length, fast-growing lines with lateral branches were selected and cultured on solidified MS medium, as seen in Fig. 3D. The hairy roots were subcultured until their fresh weight reached around 0.5g, which took five weeks to achieve, as shown in Fig. 3E. The hairy roots were then transferred into 1/2MS liquid medium (pH = 5.8), as demonstrated in Fig. 3F. After eight weeks of growth in the liquid medium, the hairy roots of *Perilla* reached a stagnation period, as illustrated in Fig. 3G and 3H. These observations help to understand the growth pattern of *P. frutescens* hairy roots during culturing, which could be useful in optimizing conditions for large-scale production of phenolic acids using these hairy root cultures.

3.2. PCR identification of hairy roots

In this study, five fast-growing perilla hairy root lines (P1-P5) were selected for identification. The DNA was isolated from the hairy roots, *A. rhizogenes* (positive control), and non-transformed sterile seedlings roots (negative control) for PCR analysis. The results showed that plasmid fragments containing genes *rolB* (423 bp) and *rolC* (622 bp) were detected in all analyzed *P. frutescens* hairy root lines and positive controls (*A. rhizogenes* C58C1 plasmid DNA). However, these fragments were not observed in the negative control (non-transformed sterile seedling roots), as seen in Fig. 4. These findings confirm the

successful transformation of the *P. frutescens* plants with the *A. rhizogenes* C58C1 strain and indicate that the *rolB* and *rolC* genes are present in the five perilla hairy root lines.

3.3. Quantitative analysis

3.3.1. analysis of the content of phenolic acids in different lines

This study also aimed to determine the contents of five phenolic acids, namely Protocatechuic aldehyde, caffeic acid, ferulic acid, RA, and ethyl caffeic acid, in the hairy roots of *P. frutescens* lines P1-P5 using HPLC analysis. The results showed that these phenolic acids exhibited different accumulation patterns in each line. Among the five identified compounds, RA was found to be the predominant compound in the extracts of the perilla hairy roots, with a content range of 4.27 to 19.08 mg/g DW. The highest production of RA was observed in the P4 line (19.08 mg/g DW), as seen in Fig. 5. The content of RA in the perilla hairy roots was about 6.4-fold higher than that reported in a previous study on the roots of the leaves of *P. frutescens* (Zhao et al., 2019). Caffeic acid was also detected in the perilla hairy roots, with content ranging from 0.14–0.49 mg/g DW, and the highest amount being observed in the P2 line. The content of ethyl caffeate ranged from 0.16–0.54 mg/g DW. Ferulic acid and protocatechualdehyde were also found in the hairy roots, with content ranging from 0.01–0.06 mg/g DW and 0.02–0.05 mg/g DW, respectively, as seen in Fig. 5. These findings suggest that the *P. frutescens* hairy root cultures can be used as a source of natural bioactive compounds for various pharmaceutical and industrial applications.

3.3.2. Effect of MeJA and SA induction on phenolic production in Perilla hairy roots

3.3.2.1 the effect of inducing days on the growth of hairy roots

The growth characteristics of *P. frutescens* hairy roots were studied by cultivating them in 1/2MS medium for 56 days, and samples were taken every seven days to analyze the fresh and dry weight of the hairy roots. The increment curves of both dry and fresh weights were obtained, as seen in Fig. 6A. From the graph, it can be observed that the growth curve of *P. frutescens* hairy roots is an "S-shaped" curve. The growth of the hairy roots is relatively slow from 0 to 14 days, followed by a logarithmic growth period from 14 to 42 days. After 42 days, the growth of the hairy roots enters a stagnant period. The fresh weight and dry weight of hairy roots growing in the plateau stage are 14 times and 0.9 times higher than those at the beginning, respectively. Therefore, the highest biomass accumulation of hairy roots occurs when they grow for six weeks. The effect of MeJA and SA inducers on the biomass accumulation of *P. frutescens* hairy roots in liquid culture was also investigated. During the first 6 days of induction treatment with MeJA or SA, the biomass of *P. frutescens* hairy roots did not change significantly, with a multiplication factor of less than 1.5. However, on the 9th day of induction treatment, their fresh weight

multiplication factor reached 3.2 and dry weight multiplication factor reached 4.7. This indicates that MeJA and SA inductions can promote the growth of *P. frutescens* hairy roots. The effect of MeJA and SA inducers on the proliferation of dry and fresh weight was not significant until 6 days before induction, after which there was a significant increase in proliferation rate. On the 9th day after treatment, the proliferation rate greatly increased, as seen in Fig. 6B,C. These findings suggest that MeJA and SA treatments could be used to enhance the growth and biomass accumulation of *P. frutescens* hairy roots in liquid culture.

MeJA treatment was found to affect the phenolic acid accumulation in *P. frutescens* hairy roots. The content of RA was significantly increased after MeJA treatment and reached its maximum (17.36 mg/g DW) on the third day, which was about 1.7-fold higher than that at 0 days, as seen in Fig. 7. Caffeic acid accumulation showed a gradual increase and reached its maximum (0.49 mg/g DW) on the ninth day after MeJA addition. The yield of ferulic acid was also increased to 5.6-fold that at 0 days and reached 0.17 mg/g DW. The highest content of protocatechualdehyde (0.08 mg/g DW) was observed on the third day after MeJA treatment, but it decreased significantly on the sixth day. After MeJA treatment, the concentration of ethyl caffeate gradually increased until the ninth day, then decreased, and reached its maximum (0.39 mg/g DW) on the sixth day. These findings demonstrate that MeJA treatment could enhance the accumulation of certain phenolic acids in *P. frutescens* hairy roots, such as RA, caffeic acid, ferulic acid, protocatechualdehyde, and ethyl caffeate. These compounds are known for their various health benefits, including anti-inflammatory, antioxidant, and antimicrobial activities. Therefore, these results suggest that MeJA-treated *P. frutescens* hairy roots could be used as a valuable source of natural bioactive compounds with potential applications in the pharmaceutical and food industries.

The addition of SA was found to affect the content of the five phenolic acids in *P. frutescens* hairy roots. Among the five detected compounds, the concentration of RA increased after treatment with SA for nine days and reached its maximum (20.92 mg/g DW), which was about 1.58-fold higher than that of the control samples, as seen in Fig. 8. SA inhibited the accumulation of caffeic acid within six days and then showed a significant enhancement on the ninth day. Finally, a 1.26-fold increase (0.63 mg/g DW) in caffeic acid was observed at nine days compared to the beginning of the experiment. The effect of SA on the content of protocatechualdehyde, ferulic acid, and ethyl caffeate showed a similar pattern as caffeic acid. A prominent decrease appeared during the first six days after treatment; subsequently, they increased on the ninth day and reached their highest levels (0.03 mg/g, 0.06 mg/g, and 0.77 mg/g DW, respectively), as shown in Fig. 8. These results imply that SA treatment could significantly alter the accumulation of certain phenolic acids, such as RA, caffeic acid, protocatechualdehyde, ferulic acid, and ethyl caffeate, in *P. frutescens* hairy roots. These compounds have been previously reported to possess various medicinal properties, including antioxidant, antimicrobial, and anti-inflammatory effects. Therefore, the SA-treated *P. frutescens* hairy roots could be of potential value in the development of natural bioactive compounds used in pharmaceutical and food industries.

4. Discussion

The use of hairy root cultures for the production of bioactive compounds in pharmaceuticals has been extensively reported in literature. For instance, studies have shown that *S. bulleyana* hairy roots can be used to produce polyphenolic compounds (Wojciechowska et al., 2020), *Trachyspermum ammi* hairy roots can produce thymol (Vamenani et al., 2020), *Gymnema sylvestre* hairy roots can produce gymnemic acid (Rajashekar et al., 2016), *Artemisia* hairy roots can produce phenol, flavonoid, sterol, and essential oil (Mottaki et al., 2019). Furthermore, *Salvia viridis* hairy roots can be utilized to produce polyphenolic compounds (Grzegorzczak-Karolak et al., 2018), *Brugmansia candida* hairy roots can produce tropane alkaloids (Cardillo et al., 2016), *Rehmannia elata* hairy roots can produce iridoid and phenylethanoid glycosides (Piątczak et al., 2019), and *Corylus avellana* hairy roots can produce saponins (Jalalipour Parizi et al., 2020). These examples demonstrate the potential of using hairy root cultures to produce a variety of bioactive compounds for multiple applications.

In our study, we successfully established a rapid and efficient system for cultivating *P. frutescens* hairy roots for the first time, allowing us to investigate the accumulation of phenolic acids in these roots. Our results revealed that compared to the original perilla plants, the cultivated hairy root showed a higher yield of valuable secondary metabolites that could serve as a consistent resource for producing pharmaceutical components. Specifically, HPLC analysis of the hairy roots demonstrated that RA was the predominant compound in the extract. The content of RA in the hairy roots was six times higher than that found in the original plants (perilla leaf) (Dai et al., 2014; Zhao et al., 2019).

Of note, only a handful of medicinal plants or hairy roots have been reported to contain higher levels of RA than perilla hairy roots. For instance, *S. viridis* hairy roots contained 35.6 mg/g DW of RA (Grzegorzczak-Karolak et al., 2018), *S. bulleyana* hairy roots contained 39.6 mg/g DW of RA (Wojciechowska et al., 2020), and *Dracocephalum moldavica* contained 38.9 mg/g DW of RA (Dastmalchi et al., 2007). In contrast, the RA content in *Ocimum basilicum* was only 1.7 mg/g of FW (Kim et al., 2006). These findings suggest that perilla hairy roots hold significant potential for RA production when compared to other medicinal plants.

Elicitors, which are compounds that induce stress-related responses and developmental processes in plants, have become increasingly popular in recent years as effective promoters of secondary metabolite production in hairy root cultures. For example, MeJA has been shown to promote the phenolic content in *Mentha spicata* (Yousefian et al., 2020), *O. basilicum* (Kim et al., 2006) and *multiorrhiza* (Xiao et al., 2009; Xing et al., 2018) as well as enhance the production of triterpenoids in hairy roots of *Centella asiatica* (Nguyen et al., 2019) and terpenoid indole alkaloids in *Rhazya stricta* (Akhgari et al., 2019). Additionally, MeJA has been found to improve tanshinones' content in *S. multiorrhiza* hairy roots (Xing et al., 2018). SA has also demonstrated potential as an elicitor for promoting secondary metabolite production in hairy roots. For example, SA has been shown to improve phenolic acid and tanshinone production in *S. przewalskii* hairy roots (Li et al., 2020) and enhance papaverine and noscapine contents in *Papaver armeniacum* hairy root culture (Sharifzadeh Naeini et al., 2021). Moreover, Ag⁺ and yeast have been found to promote tanshinone accumulation in *S. multiorrhiza* hairy root cultures (Li et al., 2016; Xiao et al., 2010).

In our study, treatment with MeJA resulted in a remarkable accumulation of RA in *P. frutescens* hairy roots on day three, with yields of 17.36mg/g DW for RA and 0.08 mg/g DW for Protocatechuic aldehyde. Caffeic acid and ferulic acid contents reached their maximum levels on day nine, while ethyl caffeate levels were increased by MeJA on day six but decreased on day nine. These findings suggest that MeJA can promote the rapid accumulation of RA in a short period of time (3 days) while inhibiting its accumulation over an extended time period. These results are consistent with previous studies conducted using *S. miltiorrhiza* hairy root cultures (Xiao et al., 2009; Xing et al., 2018) and *przewalskii* hairy root cultures (Li et al., 2020).

Our results showed that treatment with SA led to more dramatic changes in the production of phenolic compounds in *P. frutescens* hairy roots. The content of RA was found to reach its maximum level on day nine (20.92 mg/g DW). In comparison to MeJA treatment, SA treatment did not stimulate the production of Protocatechuic aldehyde and ferulic acid. On the other hand, the accumulation of caffeic acid and ethyl caffeic acid was suppressed by SA treatment on day three and day six but reached their maximum yields at the end of the treatment stage (day nine), with maximum yields of 0.63 mg/g DW and 0.77 mg/g DW (1.26-fold and 1.43-fold of the control), respectively.

The accumulation trends observed in our study suggest that SA has a promoting effect on the accumulation of RA, which is consistent with previously reported results in *S. przewalskii* hairy roots (Li et al., 2020). These findings indicate that SA could be a valuable elicitor in enhancing the yield of RA in *P. frutescens* hairy root cultures. This information contributes to the understanding of the mechanisms underlying SA-induced secondary metabolism in plants, providing insight into new approaches for plant cell culture-based bioproduction of bioactive compounds.

5. Conclusions

In summary, this study represents the first report on the establishment of *P. frutescens* hairy roots and the determination of phenolic acid accumulation in these roots. The content of RA in hairy root cultures was found to be much higher than that of the original plant, with a maximum yield of 19.084 mg/g. This finding highlights the potential of *P. frutescens* hairy roots as a source of RA production.

Furthermore, our results suggest that elicitor treatment with MeJA or SA can affect the accumulation of phenolic acids in *Perilla* hairy roots. MeJA treatment promoted RA accumulation in a short period pattern, while SA treatment showed a more continuous pattern. The maximum RA content after MeJA treatment was 17.364 mg/g at three days, while the maximum RA content after SA treatment was 20.919 mg/g at nine days. These findings demonstrate the flexible regulation strategies that can be employed for RA production in *P. frutescens* hairy root cultures. Overall, the establishment of *P. frutescens* hairy roots and the elucidation of elicitor-induced RA production provide valuable insights into plant cell culture-based bioproduction of bioactive compounds. Our results also offer a foundation for future studies aiming to explore alternative strategies for enhancing the yield of RA and other valuable metabolites using hairy root cultures.

Declarations

Authors' contributions

All authors contributed to the study conception and design. PD and YPW conceived and designed the experiments. Material preparation, data collection and analysis were performed by XYH, QS, RH, PW and MY. The first draft of the manuscript was written by YY and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

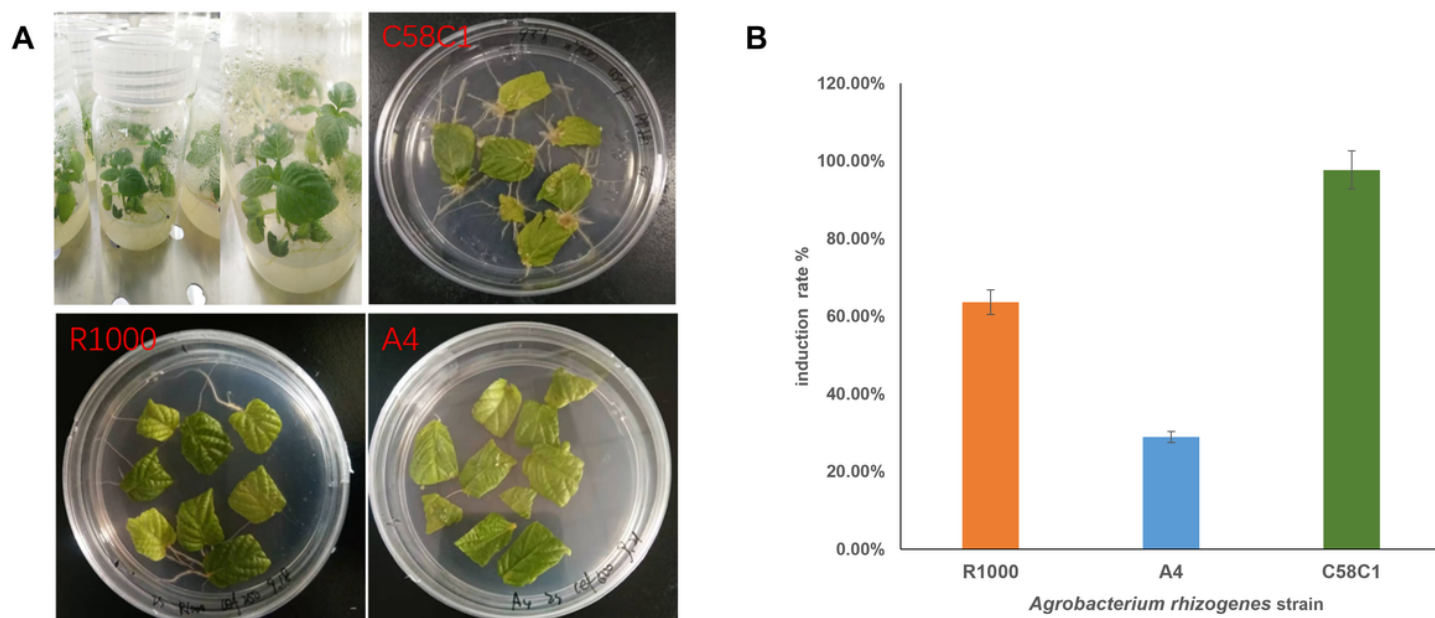


Figure 1

Leaves of *P. frutescens* infected by different strains and the induction rate of hairy roots in *P. frutescens*

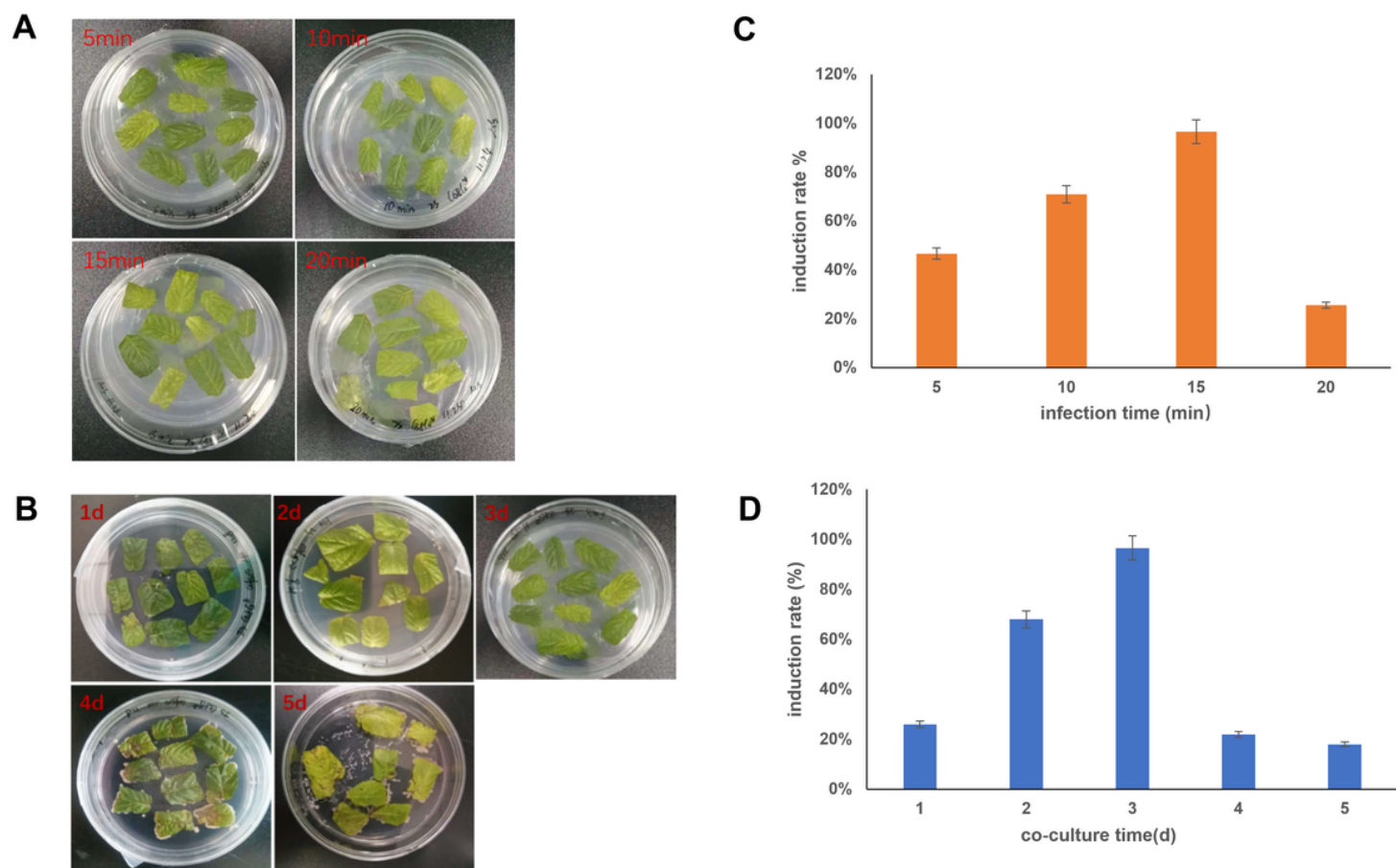


Figure 2

different infecting times and co-culture times on the induction rate of hairy roots.

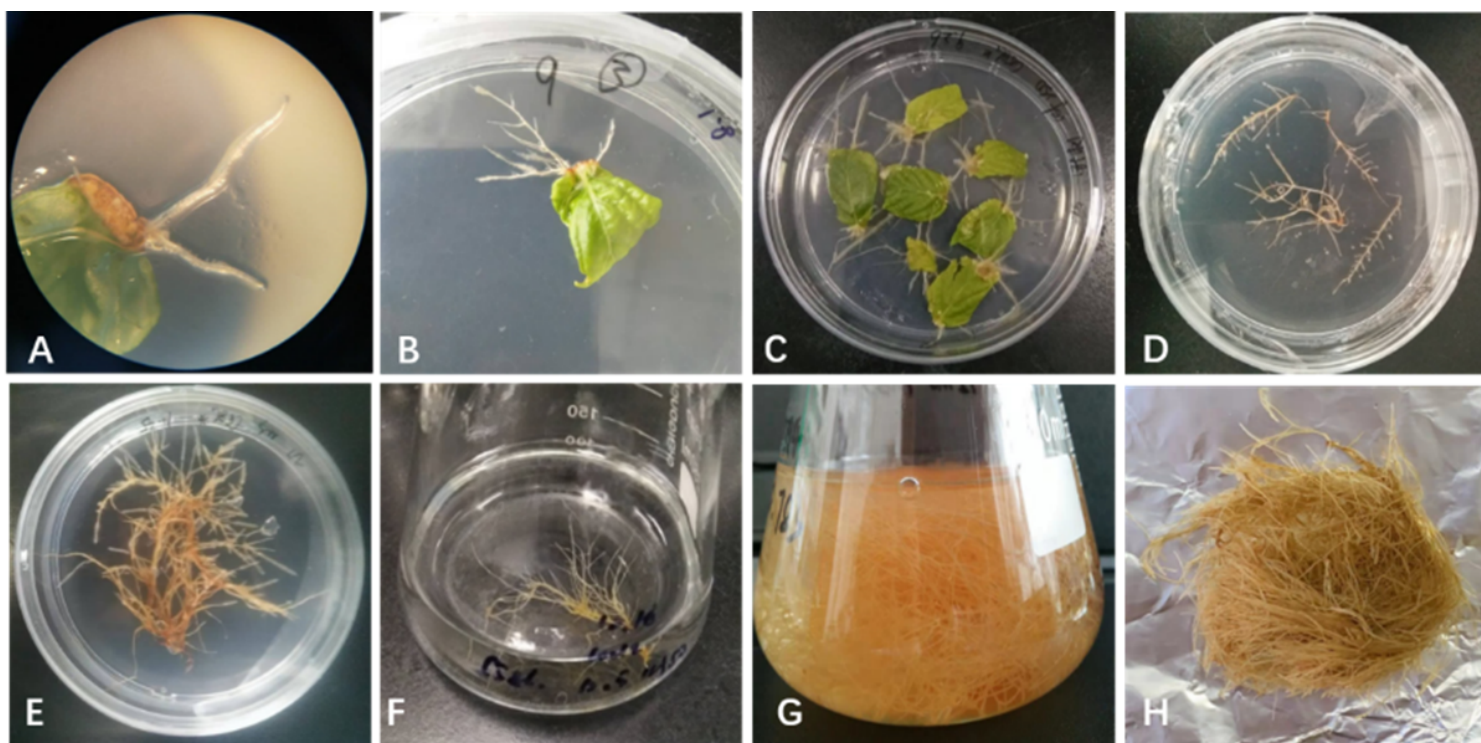


Figure 3

Hairy root induction and culture stages of *P. frutescens*. A) internode, B) hairy root induction, C) hairy roots growth two weeks after induction, D) hairy roots growth three weeks reached 5cm, E) hairy roots growth six weeks reached 0.5g, F) hairy roots transferred from solid to liquid medium, G) hairy roots growth eight weeks in the liquid medium, H) hairy roots before dried and powdered.

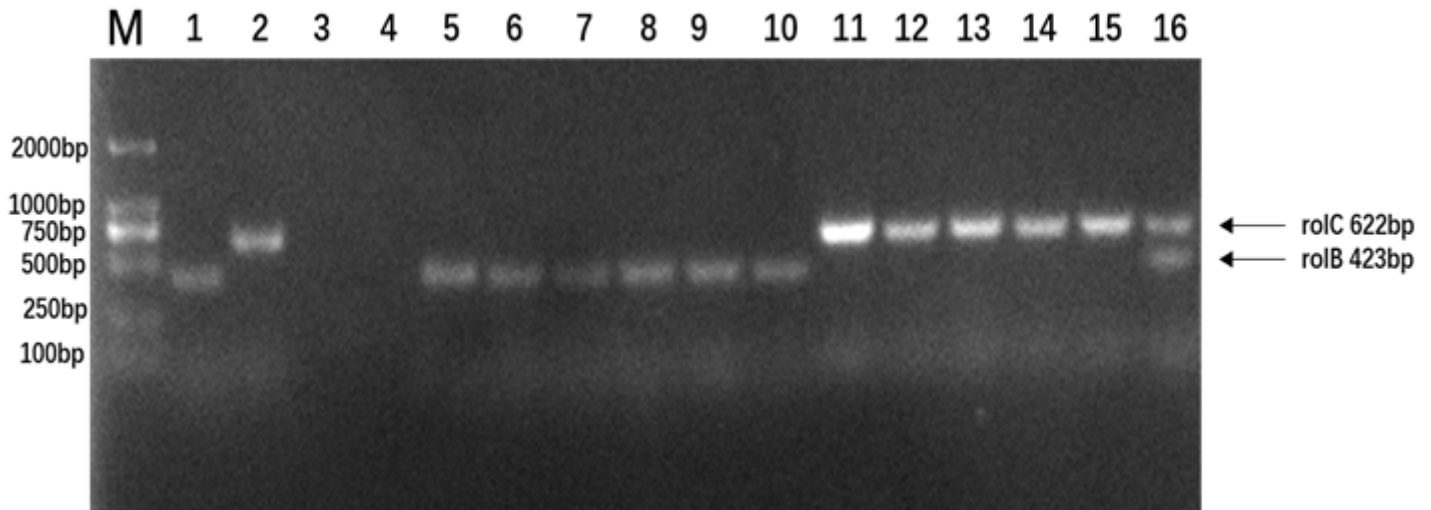


Figure 4

PCR identification of hairy roots, M is the DL2000bp marker; lanes 1 and 2 are a positive control: C58C1 plasmid DNA; lanes 3 and 4 are the negative control: perilla sterile seedling root DNA; lanes 5-16 are hairy root DNA; (Lanes 5-10 are the rolB gene, and lanes 11-16 are the rolC gene).

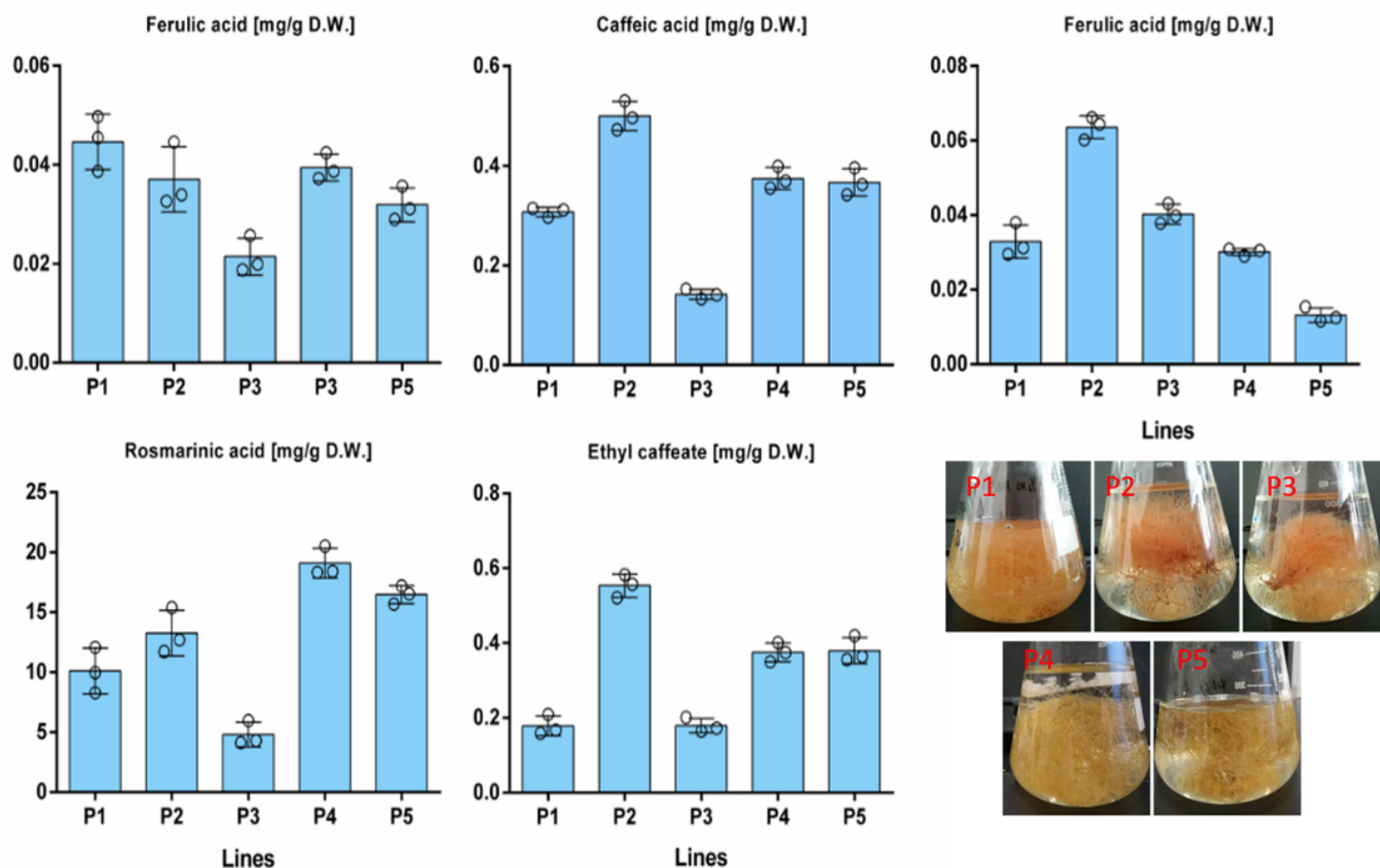


Figure 5

The contents of phenolic acids in the hairy root lines

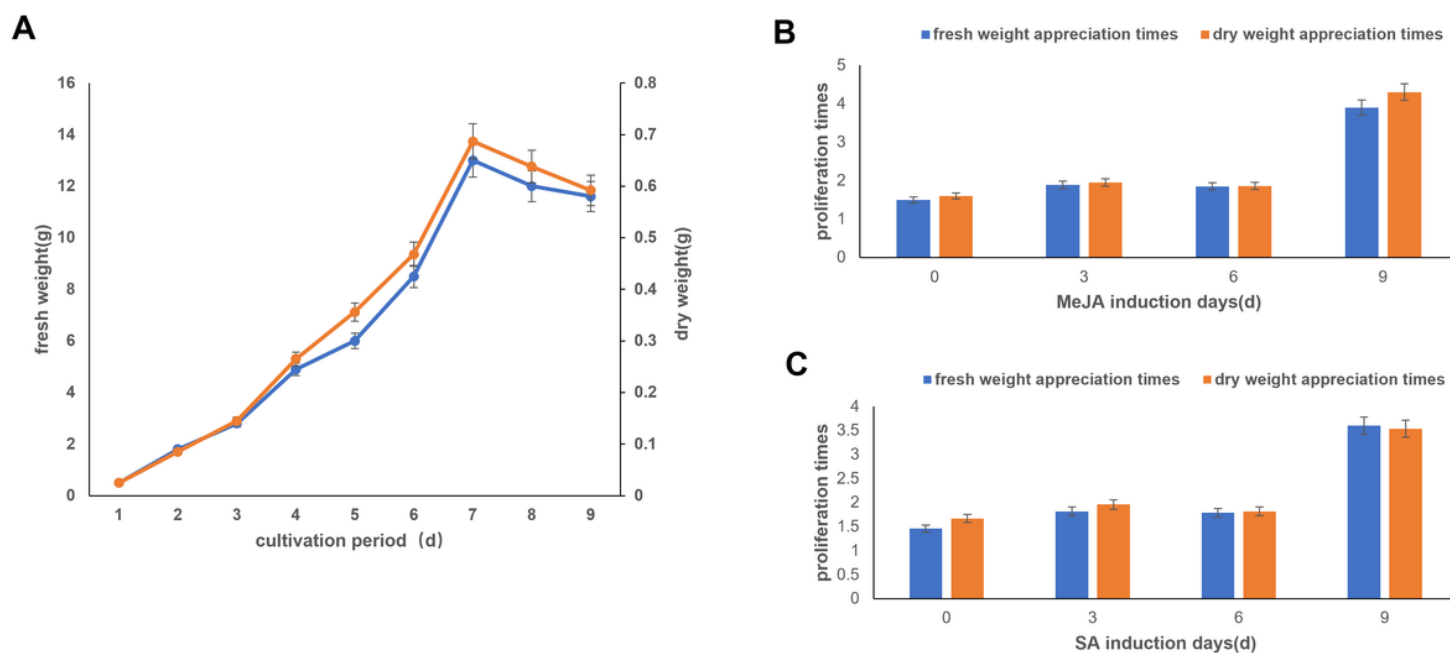


Figure 6

Growth biomass and changes in weight after treatment

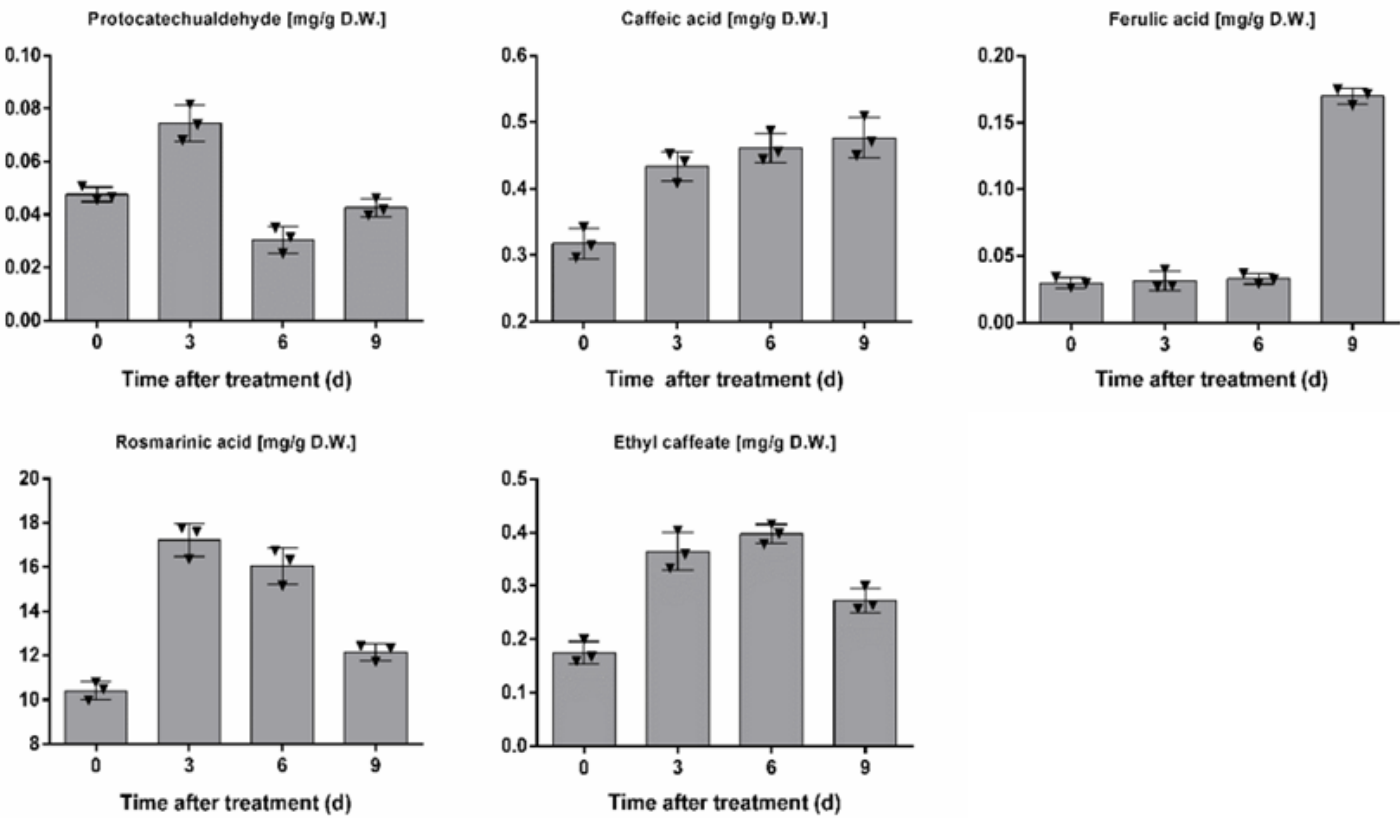


Figure 7

Effects of MeJA on the accumulation of phenolic acids in *Perilla* hairy root

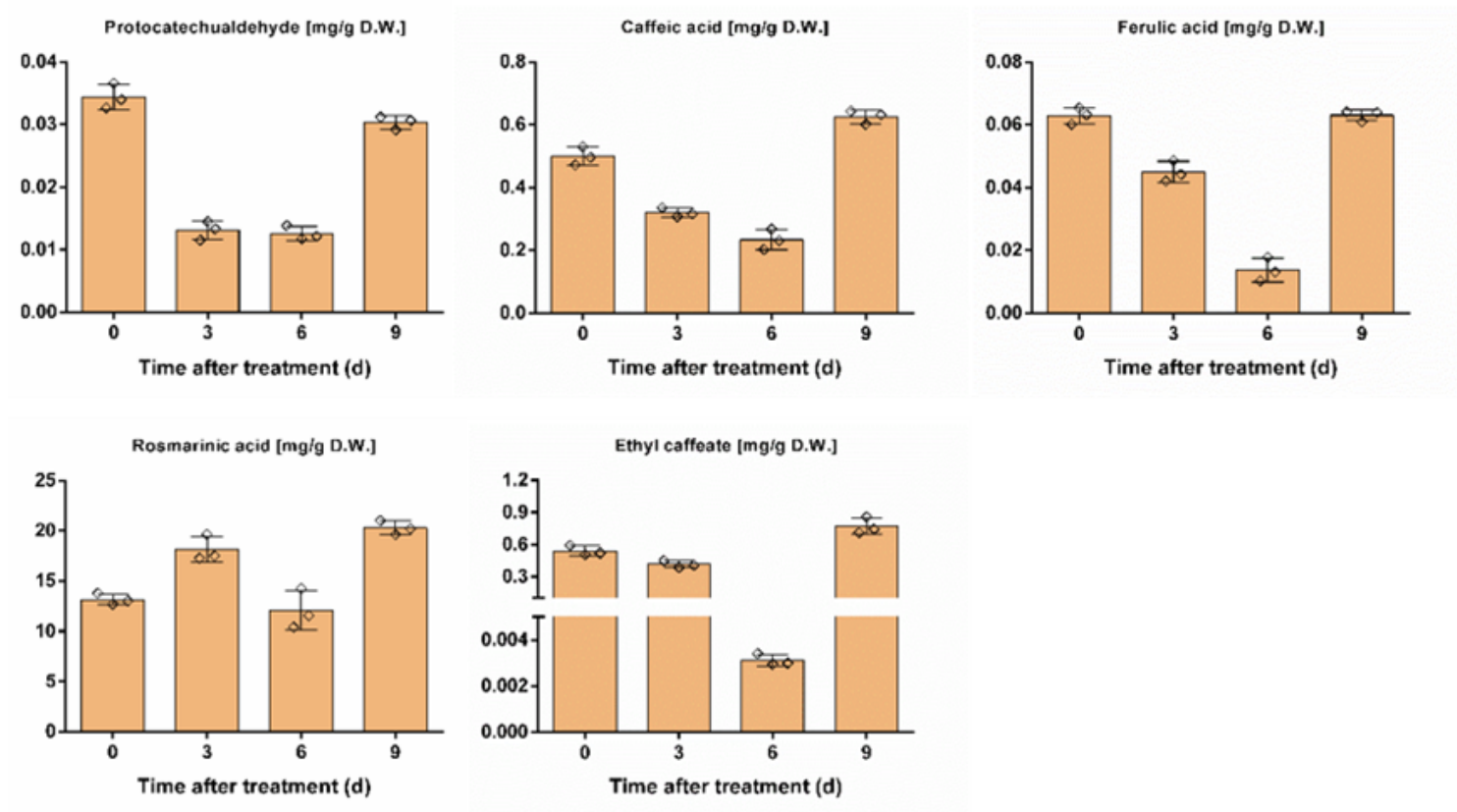


Figure 8

Effects of SA on the accumulation of phenolic acids in Perilla hairy root