Impact of Betaine Under Salinity on Accumulation of Phenolic Compounds in Safflower (*Carthamus tinctorius* L.) Sprouts

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**Abstract**

It has been assumed that abiotic stresses often lead to osmotic and ionic stress in plants either inducing or reducing secondary plant metabolites. Therefore, the influence of NaCl, glycinebetaine (betaine), and NaCl with betaine on the growth and variation in the accumulation of phenolic compounds was investigated in safflower (*Carthamus tinctorius* L.). The growth pattern of safflower sprouts was significantly influenced by these treatments. It was found that with increases in the concentration of NaCl, all growth parameters steadily decreased, but growth was markedly increased by adding different concentrations of betaine, especially at 0.5 mM, which produced the highest growth in terms of different growth parameters. High-performance liquid chromatographic (HPLC) analysis revealed changes in 7 different phenolic compounds in response to different treatments. After treatment with up to 200 mM NaCl, the levels of catechin, ferulic acid, benzoic acid, and kaempferol increased, whereas the levels of the remaining phenolic compounds, especially chlorogenic acid, and *p*-coumaric acid were reduced. Our results suggest that the growth suppression due to salinity stress is decreased in the sprouts of safflower by adding betaine.

**Keywords**
salinity, betaine, safflower sprouts, growth parameters, phenolic compounds

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Salinity stress changes several metabolic and physiological mechanisms of crops, hinders their growth and development, and finally reduces crop yields. During salinity stress, a plant's metabolism is disturbed by the accumulation of excessive sodium (Na+) and chloride ions (Cl-), inducing osmotic stress, ion imbalance, and cytotoxicity.1 Salinity responses in plants have two main phases. Initially, a decrease in growth and development occurs because of a reduction in absorption capacity of water in their root systems, an increase in water loss from their leaves, stomatal closure, and repression of cell expansion.2,4 The second phase occurs after days or weeks and leads to cell death due to delayed physiological and metabolic mechanisms, increasing cytotoxicity, and early senescence.5,6 Many physiological mechanisms, such as nutrient imbalance, reduced photosynthetic activity, and reduced detoxification of reactive oxygen species, are provoked by osmotic stress.1,5 Salinity stress can either increase or decrease the accumulation of specific types of secondary metabolites in safflower.7,9 Kim et al.10 reported that salinity stress enhances the phenylpropanoid content in tartary buckwheat. In addition, it has been reported that endogenous jasmonic acid levels in tomato plants (*Solanum lycopersicum*) are enhanced under saline conditions.11

Safflower has been cultivated worldwide as an oil crop because it usually grows well, despite a poor soil environment.12 Safflower seeds have been used in pharmaceuticals and...
cooking oil because they contain abundant dietary fibers and phenolic compounds.\textsuperscript{13,14} It has also been reported that oil-rich cultivars of safflower oil contain abundant amounts of tocopherol and polyunsaturated fatty acids.\textsuperscript{15} However, most plant seeds have either indigestible or toxic compounds in the epidermal cells or seed coats for defense against animals.\textsuperscript{16} Therefore, some crops, such as beans, buckwheat, broccoli, cabbages, and radishes, are ingested as sprouts instead of seeds because the indigestible substances in the seed coat or epidermal cells are removed during sprout growth.\textsuperscript{17} Several researchers have investigated the nutritional factors of safflower sprouts during the germination stage, such as vitamin C antioxidant activity, crude protein, and fat.\textsuperscript{18,19}

Phenylpropanoids are the largest group of organic compounds, derived from the 6-carbon phenyl group, that are biosynthesized from tyrosine and phenylalanine.\textsuperscript{20} These groups are located throughout the plant domain and perform many physiological functions such as mediating plant-pollinator interactions and protecting against pathogens, ultraviolet light, and herbivores.\textsuperscript{21} Flavonoids, a big subgroup of phenylpropanoids, primarily comprise compounds that are based on flavonols, anthocyanins, and proanthocyanins.\textsuperscript{22,23} The antioxidant properties play a dynamic physiological role when plants interact with biological and abiotic environments.\textsuperscript{22,24-26}

Compatible solutes, such as betaine and proline, are small organic molecules with low toxicity and neutral charge at high concentrations that help organisms survive extreme osmotic stress and act as osmolytes.\textsuperscript{27} Glycinebetaine (betaine), a quaternary ammonium compound, is an N-methyl-substituted derivative of glycine. It is also a functional compound that regulates stress responses, protects proteins and enzymes, and maintains cell osmotic pressure. It is generally found in a variety of microorganisms, animals, and higher plants such as vascular and flowering plants.\textsuperscript{28,29} Accumulation of betaine increases stress tolerance in several crop species such as sorghum, spinach, and barley.\textsuperscript{30} However, the influences of betaine treatments on the accumulation of phenolic compounds and salt stress responses have not been fully investigated, especially for safflower. Hence, in the present study, we evaluated the effects of betaine treatments on the growth and accumulation of phenolic compounds in safflower sprouts under NaCl stress.

Results

Variations in Growth and Accumulation of Phenolic Compounds in Safflower Sprouts in Response to NaCl Treatments

Different concentrations of NaCl (0, 50, 100, 200, and 300 mM) were applied to investigate the growth patterns of safflower sprouts. All the growth parameters were significantly influenced by the application of all NaCl concentrations (Figure 1), with all decreasing regularly with increasing concentrations. The fresh weight of sprouts was 11.8%, 33.8%, 54.4%, and 79.4% lower in the treatments with 50, 100, 200, and 300 mM NaCl, respectively, compared with no use of NaCl (control). Shoot lengths were decreased by 14.4%, 33.3%, 47.3%, and 80.4% when treated with 50, 100, 200, and 300 mM NaCl, respectively, compared to the control treatment. Root lengths were decreased by all the NaCl treatments, exhibiting lower values of 19.5, 29.0, 45.7, and 76.2% with 50, 100, 200, and 300 mM NaCl, respectively, compared to the control treatment. The germination rate of sprouts decreased regularly with increasing NaCl concentration, with values of 91.1, 90.0, 84.4, 75.6% and 55.6% with 0, 50, 100, 200, and 300 mM NaCl treatments, respectively.

In the sprouts of safflower, 7 phenolic compounds (gallic acid, catechin, chlorogenic acid, p-coumaric acid, ferulic acid, benzoic acid, and kaempferol) were detected in different amounts for the different concentrations of NaCl treatments (Table 1). Overall, the content of phenolic compounds differed with the concentration of salt treatment. Treatment with up to 100 mM NaCl resulted in increased levels of catechin, ferulic acid, benzoic acid, and kaempferol, whereas those of gallic acid, chlorogenic acid, and p-coumaric acid were reduced.
Increases in the concentration of NaCl above 200 mM decreased the concentration of all phenolic compounds in comparison with the control, except for kaempferol. The levels of catechin and total phenolic compounds were the highest after treatment with 100 mM NaCl. Treatment with 100 mM NaCl led to 9.9%, 19.7%, 22.2%, and 22.2% higher catechin contents than those with 50 mM NaCl, control, 200 mM NaCl and 300 mM NaCl treatments, respectively. Treatment with 100 mM NaCl led to 8.6%, 5.9%, 11.8%, and 31.8% higher total phenolic compound contents than those of the control, and those treated with 50 mM NaCl, 200 mM NaCl and 300 mM NaCl, respectively.

Seven phenolic compounds were detected after treatment with different concentrations of betaine (0.1, 0.5, 1, 5, and 10 mM) (Table 2). Their levels differed with the concentrations of betaine, but tended to be lower than that of the control. For all treatments, the contents of both ferulic acid and \(p\)-coumaric acid remained almost similar. The highest total content of phenolic compounds was found in the control, followed by treatment with 1 mM betaine, and the lowest was observed in the treatment with 5 mM betaine. The levels of gallic acid tended to increase as the betaine concentration increased, but the levels of catechin, chlorogenic acid, benzoic acid, and kaempferol did not show a unique trend.

### Table 2. Content of Phenolic Compounds (mg/g Dry Wt.) in Safflower Sprouts After Different NaCl Treatments.

| Phenolic compounds | Control | NaCl 50 | NaCl 100 | NaCl 200 | NaCl 300 |
|--------------------|---------|---------|---------|---------|---------|
| Gallic acid        | 12.4 ± 1.2\(^{a1}\) | 6.34 ± 0.1\(^{e}\) | 7.9 ± 0.2\(^{b}\) | 6.6 ± 0.3\(^{c}\) | 4.8 ± 0.23\(^{d}\) |
| Catechin           | 654.0 ± 46.2\(^{a}\) | 728.7 ± 18.8\(^{b}\) | 805.8 ± 56.1\(^{a}\) | 632.9 ± 14.6\(^{b}\) | 633.5 ± 22.0\(^{a}\) |
| Chlorogenic acid   | 167.1 ± 1.4\(^{a}\) | 91.7 ± 7.0\(^{b}\) | 109.8 ± 1.3\(^{a}\) | 88.1 ± 13.6\(^{b}\) | 109.6 ± 4.0\(^{a}\) |
| \(p\)-coumaric acid| 100.1 ± 4.0\(^{a}\) | 86.2 ± 6.7\(^{b}\) | 72.4 ± 3.0\(^{e}\) | 52.3 ± 5.9\(^{d}\) | 34.8 ± 0.6\(^{c}\) |
| Ferulic acid       | 64.1 ± 1.8\(^{a}\) | 56.7 ± 0.8\(^{b}\) | 73.3 ± 8.6\(^{a}\) | 63.4 ± 2.8\(^{b}\) | 27.3 ± 1.6\(^{c}\) |
| Benzoic acid       | 261.6 ± 1.7\(^{ab}\) | 281.2 ± 12.7\(^{a}\) | 282.7 ± 10.2\(^{c}\) | 243.1 ± 25.8\(^{bc}\) | 219.6 ± 11.6\(^{c}\) |
| Kaempferol         | 824.7 ± 21.3\(^{d}\) | 886.3 ± 9.0\(^{b}\) | 911.8 ± 0.8\(^{c}\) | 938.1 ± 11.4\(^{a}\) | 688.5 ± 8.3\(^{e}\) |
| Total              | 2084.0 ± 64.6\(^{cb}\) | 2137.3 ± 15.8\(^{b}\) | 2263.7 ± 62.4\(^{a}\) | 2024.5 ± 59.9\(^{e}\) | 1718.1 ± 27.3\(^{bc}\) |

\(^{1}\)Values followed by different letters within a row indicate a significant difference (\(P < 0.05\)) between areas for that parameter using DMRT (Duncan's multiple range test; \(n \geq 3\), mean ± SD).

Growth Behavior and Accumulation of Phenolic Compounds in Safflower Sprouts as Influenced by Concentrations of Betaine

Several concentrations of betaine (0.1, 0.5, 1, 5, and 10 mM) were tested for their effect on growth, that is, shoot fresh weight, and shoot and root length of safflower sprouts. A control group without betaine was used for comparison. The growth of safflower sprouts was slightly increased with the addition of betaine. A slight increase in growth of safflower sprouts was noted within the range of 0.1 and 0.5 mM betaine, but then growth declined as betaine concentration increased between 1 and 10 mM (Figure 2). It is noteworthy that the fresh weight, shoot length, and root length of safflower sprouts were higher with all concentrations of betaine (0.1-10 mM) in comparison with the control. The fresh weight of sprouts was 11.1% higher with both 0.1 and 0.5 mM betaine treatments than the control. Similarly, a significant increasing trend for shoot length was reported from 0.1–0.5 mM betaine, but, after that, it started to decline from 1 to 10 mM (Table 2). The shoot length was higher than that of the control up to a concentration of 10 mM. The highest shoot length was reported after treatment with 0.5 mM betaine, which showed a 15.5% higher shoot length than the control. A similar trend to that of shoot length was denoted for root length; the highest root length was recorded for 0.5 mM betaine, exhibiting a 10.8% higher root length than that of the control. The germination rate remained almost similar.
Combined Effect of 200 mM NaCl with Different Concentrations of Betaine on Sprout Growth and Accumulation of Phenolic Compounds

The combined application of 200 mM NaCl with different concentrations of betaine influenced the growth of safflower sprouts (Figure 3). However, none of the combined treatments enhanced a positive response in any of the growth parameters except for root length in a few instances. A slight increase in root length of 7.6% and 14.9% was observed with the treatment with 200 mM NaCl in combination with 0.1 and 0.5 mM betaine, respectively. In addition to these combinations, root lengths decreased by 2.7, 28.5, and 46.9% when combined with the treatment with 200 mM NaCl plus 1, 5, and 10 mM betaine, respectively. Shoot weight and shoot length were reduced by the application of NaCl and betaine. Moreover, treatment with 200 mM NaCl with both 5 and 10 mM betaine produced 33.3% higher gallic acid than the control. The accumulation of catechin was higher than that of the control group. The trend for shoot length was similar to that of fresh weight. The shoot length decreased by 47.2%, 35.1%, 44.3%, 50.1%, and 53.9% after treatment with 200 mM NaCl along with 0.1, 0.5, 1, 5, and 10 mM betaine, respectively, compared to the control. When betaine was added under NaCl stress, the germination rate was increased. It was 91.1% in the control group and 75.6% in the 200 mM NaCl group, but for the 200 mM NaCl groups catechin showed a tendency to increase as the concentration of betaine was lowered. The accumulation of catechin in the group treated with 200 mM NaCl with 0.1 mM betaine was higher than that of the control group. The levels of most of the phenolic compounds (chlorogenic acid, \( p \)-coumaric acid, ferulic acid, and benzoic acid), but not those of gallic acid, catechin, and kaempferol, were higher in the control group.

| Phenolic compounds | Control | Betaine 0.1 | Betaine 0.5 | Betaine 1.0 | Betaine 5.0 | Betaine 10.0 |
|--------------------|---------|-------------|-------------|-------------|-------------|--------------|
| Gallic acid        | 11.3 ± 1.0\(^{a}\) | 9.7 ± 0.9\(^{d}\) | 13.9 ± 2.7\(^{d}\) | 16.6 ± 1.6\(^{b}\) | 21.2 ± 1.3\(^{a}\) | 22.7 ± 2.3\(^{a}\) |
| Catechin           | 808.5 ± 17.6\(^{a}\) | 733.3 ± 39.8\(^{b}\) | 742.5 ± 12.7\(^{b}\) | 821.4 ± 4.4\(^{a}\) | 720.9 ± 32.2\(^{b}\) | 807.1 ± 12.4\(^{a}\) |
| Chlorogenic acid   | 135.3 ± 16.2\(^{a}\) | 138.3 ± 20.0\(^{b}\) | 120.7 ± 8.8\(^{b}\) | 140.3 ± 12.8\(^{a}\) | 134.3 ± 3.0\(^{a}\) | 104.1 ± 0.4\(^{b}\) |
| \( p \)-coumaric acid | 35.8 ± 3.6\(^{a}\) | 35.3 ± 2.0\(^{b}\) | 39.8 ± 3.5\(^{a}\) | 36.8 ± 1.5\(^{a}\) | 35.4 ± 0.5\(^{a}\) | 30.8 ± 2.0\(^{b}\) |
| Ferulic acid       | 91.3 ± 4.0\(^{a}\) | 89.5 ± 4.5\(^{b}\) | 92.2 ± 0.5\(^{b}\) | 93.4 ± 1.5\(^{a}\) | 87.5 ± 4.1\(^{b}\) | 87.0 ± 1.8\(^{a}\) |
| Benzoic acid       | 241.0 ± 33.8\(^{a}\) | 241.6 ± 14.6\(^{a}\) | 238.7 ± 10.2\(^{a}\) | 260.9 ± 8.3\(^{a}\) | 256.4 ± 10.6\(^{a}\) | 237.0 ± 11.9\(^{a}\) |
| Kaempferol         | 984.8 ± 108.4\(^{a}\) | 842.5 ± 89.1\(^{b}\) | 820.5 ± 100.7\(^{a}\) | 721.9 ± 73.6\(^{b}\) | 678.6 ± 25.3\(^{b}\) | 777.7 ± 135.8\(^{b}\) |

Total: 2308.0 ± 165.8\(^{a}\) 2090.3 ± 149.4\(^{a}\) 2063.8 ± 84.8\(^{b}\) 2091.3 ± 71.7\(^{ab}\) 1934.3 ± 34.1\(^{b}\) 2066.4 ± 152.9\(^{b}\)

\(^{1}\)Values followed by different letters within a row indicate a significant difference \((P < 0.05)\) between areas for that parameter using DMRT (Duncan's multiple range test; \(n \geq 3\), mean ± SD).

Figure 3. Growth characteristics of safflower sprouts when treated with NaCl 200 mM and different betaine concentrations (mM). Different letters indicate significant differences \((P < 0.05)\) between areas for that parameter using Duncan's multiple range test \((n \geq 15\), mean ± SD). (A) Treated safflower sprouts; (B) Fresh weights; (C) Shoot lengths; (D) Root lengths; (E) Germination rate.
Table 3. Content of Phenolic Compounds (mg/g Dry Wt.) in Safflower Sprouts Grown in NaCl 200 mM with Different Betaine Concentrations.

| Phenolic compounds | Control | NaCl 200 mM | NaCl 200 + Betaine 0.1 mM | NaCl 200 + Betaine 0.5 mM | NaCl 200 mM + betaine 1.0 mM | NaCl 200 mM + betaine 5.0 mM | NaCl 200 mM + betaine 10.0 mM |
|--------------------|---------|-------------|--------------------------|--------------------------|-----------------------------|-----------------------------|-------------------------------|
| Gallic acid        | 17.5 ± 1.4d | 20.0±3.1cd    | 18.9±1.2cd              | 20.0±2.3cd              | 23.1 ± 1.7bc               | 25.2 ± 1.6b                 | 34.8 ± 3.5b                   |
| Catechin           | 69.5 ± 9.8d | 64.3±2.07d    | 94.2±5.30d              | 79.1±7.22d              | 719.4±15.6c               | 735.9±15.8c                | 722.3±19.0f                   |
| Chlorogenic acid   | 176.7 ± 7.8e | 95.3±6.8b     | 91.7±3.1d               | 89.2±2.9e               | 83.0±2.2e                  | 87.4±2.9b                  | 78.1±1.3f                     |
| p-coumaric acid    | 58.2 ± 2.1c | 32.5±1.3c     | 38.2±2.2b               | 38.7±0.3b               | 34.5±1.9b                 | 38.3±2.9b                  | 33.2±2.0f                     |
| Ferulic acid       | 106.6 ± 7.4c | 73.9±2.7cd    | 75.9±5.4cd              | 78.8±5.2cd              | 69.8±3.3d                 | 80.7±3.4c                  | 71.3±1.1d                     |
| Benzoic acid       | 623.6 ± 48.4c | 461.9±97.7c   | 498.5±47.4c             | 500.6±39.0c             | 478.3±33.4c               | 554.0±33.2c                | 526.6±18.0c                   |
| Kaempferol         | 663.3 ± 130.3bc | 750.5±125.3bc | 809.6±201.5c            | 868.1±150.2bc           | 657.0±65.9bc              | 672.5±56.5bc               | 590.9±73.8b                   |
| Total              | 2285.7 ± 155.8c | 2078.5±174.8c | 2353.5±130.7c           | 2203.1±129.9bc          | 2065.3±60.1bc             | 2194.1±28.1bc              | 2057.3±59.7b                  |

Values followed by different letters within a row indicate a significant difference (P < 0.05) between areas for that parameter using DMRT (Duncan’s multiple range test; n ≥ 3, mean ± SD).

Discussion

The response of NaCl and betaine at different concentrations, and a combination of 200 mM NaCl and several concentrations of betaine on growth indices such as fresh weight, shoot length, and root length of safflower sprouts and variation in the phenolic compounds were investigated in this study. Betaines are used to adjust the osmotic balance, which generally accumulate in different cellular compartments. It is possible that secondary plant metabolites either increase or decrease in response to different abiotic stresses in plants. In our current study, the growth pattern changed significantly under different treatments. We observed that with increases in the concentration of NaCl, all growth parameters steadily decreased.

Several previous studies have shown a significant reduction due to salinity in the growth of different plant species, including *Fagopyrum tataricum*, *Triticum aestivum*, *Sesamum indicum*, *Carthamus tinctorius*, *Vigna radiata*, *Cassia angustifolia*, *pachyrhizus*, and *Pisum sativum*. In *C. tinctorius*, the total flavonoid content was gradually increased by increasing the salinity concentration. Similarly, in this study, the total phenolic content was gradually increased with up to 100 mM NaCl and then decreased at the highest salinity concentration. It was previously reported by Kim et al. that overall phenolic compounds were lower after treatment of buckwheat sprouts with different NaCl concentrations compared to those without NaCl (control). They also reported that 50 mM NaCl treatment slightly increased a few phenolic compounds in comparison to others. In another study, Cuong et al. asserted that NaCl, especially at concentrations of 50, 100, and 200 mM, enhanced the levels of epicatechin, but reduced the levels of catechin, benzoic acid, and quercetin in tartary buckwheat sprouts when compared with the control treatment without NaCl. The findings of this study coincide with those of Kim et al. and Cuong et al., as we found that treatment with up to 200 mM NaCl enhanced the levels of accumulation of the following phenolic compounds: 4-hydroxybenzoic acid, catechin, ferulic acid, and benzoic acid, but decreased the amounts of caffeic acid, chlorogenic acid, and p-coumaric acid.

Kim et al. reported a significant increase in the growth of tartary buckwheat sprouts after application of 0.1-1.0 mM betaine, which is supported by our current findings, whereas the addition of different concentrations of betaine, especially at 0.5 mM, enhanced the growth of sprouts of safflower. However, at higher concentrations (300 mM NaCl, 10 mM betaine, and 200 mM NaCl +10 mM betaine) the concentration of most phenolic compounds was significantly decreased in safflower sprout. A similar result was obtained in tartary buckwheat seedlings and wheat sprouts when treated with higher concentrations of NaCl and betaine. Previously, it has been reported that with salinity an exogenous application of betaine showed a significant positive influence on plant growth and accumulation of secondary metabolites. The addition of betaine not only enhanced the growth of tartary buckwheat sprouts, but increased it in wheat sprouts, while shoot weight of Pokkali rice, and shoot and root weight of IR-28 rice that had been reduced under salinity stress, were enhanced with the application of exogenous betaine. It is noteworthy that the application of betaine improved the stress tolerance of several crop species such as sorghum, spinach, and barley under salt and drought stress, as reported by Ashraf and Foolad. In addition, several researchers have found positive salt stress responses to betaine in different plants such as rice, *triticum* *sativum*, maize, *wheat*, *ryegrass*, *mung bean*, and *tomato*. Our current findings, in which exogenous application of betaine influenced the accumulation of phenolic compounds in safflower sprouts, correspond to these.

None of the treatment combinations led to a significant increase in the overall growth of safflower sprouts, except for a few instances of root length. Here, in this study, 7 different phenolic compounds were detected in response to different treatments. Treatment with NaCl up to 200 mM increased the contents of catechin, benzoic acid, and kaempferol. On the other hand, the amounts of gallic acid, chlorogenic acid, and p-coumaric acid were decreased. There was no unique trend for the phenolic compounds due to betaine treatments; the control group contained the highest total amount of phenolic compounds. In the NaCl with betaine...
treatments, the accumulation of total phenolics was highest after treatment with 200 mM NaCl combined with 0.1 mM betaine. Especially the levels of gallic acid, catechin, and kaempferol were higher than in the control.

Previous researchers have noted that moderate salinity resulted in the enhancement of phenolic compounds in red peppers (Capsicum annuum L.), whereas the reverse results were reported in green and turning peppers. Exogenous application of NaCl has been reported to either enhance or reduce the accumulation of secondary metabolites. In addition, from our results, it is shown that phenolic compounds were changed by salinity stress. This result was similar to those of plants such as Cynara scolymus, Fagopyrum esculentum, Thymus daenensis, and Thymus vulgaris exposed to salinity stress.

Furthermore, betaine has been reported to reduce the proline content in salt-stressed perennial ryegrass. The protein content in IR-28 rice was reduced under saline conditions, while an increasing trend was observed after betaine application. Our results, which show that salinity increases the accumulation of phenolic compounds in sprouts of safflower, are consistent with the findings reported earlier, including those relating to NaCl treatment of Solanum nigrum, maize, and red pepper. This suggests that the variable contents of phenolic compounds due to abiotic stress, especially salt stress or betaine stress, or their combined effect may be species-dependent.

Conclusions

The growth of sprouts of safflower decreased steadily with increasing concentrations of NaCl. On the other hand, the vegetative growth of safflower sprouts increased greatly in response to concentrations of betaine, especially at 0.5 mM, which showed the highest growth. Treating with NaCl up to 200 mM led to increases in the contents of 3 phenolic compounds, namely, catechin, benzoic acid, and kaempferol. On the other hand, the amounts of gallic acid, chlorogenic acid, and p-coumaric acid were decreased. Treatment with NaCl and betaine led to the accumulation of total phenolic compounds, with 200 mM NaCl combined with 0.1 mM betaine resulting in values higher than those of the other treatments; the values tended to decrease with increasing concentrations of betaine. Studies have shown that secondary metabolites in several plant species differ under abiotic stress. Our findings provide additional information for the accumulation of phenolic compounds under abiotic stressors in the sprouts of safflower, which might help to explain the increased levels of secondary metabolites in other plant species.

Experimental

Plant Materials

Safflower seeds were purchased from Asia Seed Co. Ltd. (Seoul, Korea). Thirty safflower seeds were sown in a plastic pot (12 cm × 12 cm) containing vermiculite; seeds were planted at a depth of 2 cm; 3 pots were used for the control and each experimental group. All experimental plants were kept in a plant growth chamber maintained at 25 °C under a 16 h light/8 h dark program. To determine the optimal concentration of NaCl for safflower sprouts, 50, 100, 200, and 300 mM NaCl aqueous solution was added every day for about 2 weeks. Also, to determine the response of betaine, 0.1, 0.5, 1, 5, and 10 mM betaine hydrochloride (Sigma-Aldrich Co., St. Louis, MO, USA) aqueous solution was applied every day for 2 weeks. Finally, betaine along with NaCl was applied to safflower seeds every day with 200 mM NaCl aqueous solution and 0.1, 0.5, 1, 5, or 10 mM betaine in 200 mM NaCl aqueous solution for 2 weeks to determine the combined effect. Treated seeds were grown for 2 weeks and then sprouts were harvested. The fresh weight, shoot length and root length of the treated samples were measured. Measurement were made in triplicates (in each replicate 5 plantlets were measured). In total, 15 plantlets were taken for the growth measurements. The germination rate was also investigated.

Phenylpropanoid Extraction

Immediately after collection, the harvested samples were frozen using liquid nitrogen and lyophilized at -70 °C for 72 h to extract the phenolic compounds. The lyophilized samples were crushed to a fine powder using a mortar and pestle. A 100 mg sample was taken to which 2 mL of 80 % methanol was added. The mixture was vortexed vigorously for 1 min, and sonicated for 1 h at 36 °C. Then, the samples were centrifuged at 12,000 rpm at 4 °C for 15 min, the supernatants transferred to new tubes, filtered through 0.45 μm Whatman No. 42 filter paper, and moved to vials for HPLC analysis.

HPLC Analysis of Phenylpropanoid Content

For HPLC analysis, a Futecs model NS-4000 apparatus (Futecs Co. Ltd., Daejeon, Korea) was used with a 250 mm × 4.6 mm, 5 μm C18 column (RSTech Co. Ltd., Daejeon, Korea). The following conditions were applied: column temperature 30 °C, UV wavelength set at 280 nm, volume of injection 10 μL, and flow rate 1.0 mL/min. The mobile phase consisted of (A) water:acetic acid (99.85:0.15 v/v) and (B) 100% methanol. The opening composition of the mobile phase was as follows: 5% solvent B, followed by a linear gradient from 5%–80% solvent B over 93 min, then holding at 5% solvent B for an additional 5 min. Calculation of phenolic compound contents was based on the calibration curve and the peak area of the standard compounds for each sample. The analysis was repeated 3 times. The calibration curves equation, coefficient of determination, limit of detection (LOD), and limit of quantitation (LOQ) of the standard compounds are presented in Table 4. All standards used in this work were purchased from Sigma-Aldrich (Sigma-Aldrich Co., St Louis, MO, USA) and the purity of all was ≥95%. All standards were dissolved in ethanol. For quantification and identification, a spike test was performed by...
adding the standard to the extract sample. HPLC analysis was performed in triplicate.

**Statistical Analysis**

All the growth data are presented as the mean ± standard deviation of 15 repeats, and the data for phenolic compounds are presented as the mean ± standard deviation of 3 replicates. Both growth and HPLC data were analyzed using the software IBM SPSS statistics 24 (IBM Corp., Armonk, NY, USA). Statistical significance was evaluated by analysis of variance (ANOVA) using Duncan's multiple range test (DMRT) at $\text{P} \leq 0.05$.

**Declaration of Conflicting Interests**

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| Phenolic compound   | Calibration curve equation$^1$ | Coefficient of determination ($R^2$) | LOD (µg/mL) | LOQ (µg/mL) |
|---------------------|---------------------------------|-------------------------------------|-------------|-------------|
| Gallic acid         | $y = 114470.36 x - 16818.77$    | 0.9996                              | 1.441       | 4.368       |
| Catechin            | $y = 28025.39 x - 2223.87$      | 0.9997                              | 0.717       | 2.173       |
| Chlorogenic acid    | $y = 52135.70 x - 57351.97$     | 0.9991                              | 1.672       | 5.068       |
| $p$-coumaric acid   | $y = 172376.91 x - 2288.50$     | 0.9991                              | 0.220       | 0.665       |
| Ferulic acid        | $y = 115029.24 x - 16713.48$    | 0.9995                              | 0.345       | 1.044       |
| Benzoic acid        | $y = 16424.67 x +1917.49$       | 0.9996                              | 1.486       | 4.503       |
| Kaempferol          | $y = 67783.77 x - 6708.52$      | 0.9995                              | 0.529       | 1.602       |

$^1y$ and $x$ are the peak area and concentration of the analyte, respectively.

### Table 4. Calibration Curve Equation, Coefficient, LOD, and LOQ of Standards

- Gallic acid: $y = 114470.36 x - 16818.77$, $R^2 = 0.9996$, LOD = 1.441 µg/mL, LOQ = 4.368 µg/mL
- Catechin: $y = 28025.39 x - 2223.87$, $R^2 = 0.9997$, LOD = 0.717 µg/mL, LOQ = 2.173 µg/mL
- Chlorogenic acid: $y = 52135.70 x - 57351.97$, $R^2 = 0.9991$, LOD = 1.672 µg/mL, LOQ = 5.068 µg/mL
- $p$-coumaric acid: $y = 172376.91 x - 2288.50$, $R^2 = 0.9991$, LOD = 0.220 µg/mL, LOQ = 0.665 µg/mL
- Ferulic acid: $y = 115029.24 x - 16713.48$, $R^2 = 0.9995$, LOD = 0.345 µg/mL, LOQ = 1.044 µg/mL
- Benzoic acid: $y = 16424.67 x +1917.49$, $R^2 = 0.9996$, LOD = 1.486 µg/mL, LOQ = 4.503 µg/mL
- Kaempferol: $y = 67783.77 x - 6708.52$, $R^2 = 0.9995$, LOD = 0.529 µg/mL, LOQ = 1.602 µg/mL

$^1y$ and $x$ are the peak area and concentration of the analyte, respectively.
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