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THE INFLUENCE OF BIOSTIMULANTS ON THE CONTENT OF PHENOLIC COMPOUNDS IN BROCCOLI HEADS DURING SHORT-TERM STORAGE AT ROOM TEMPERATURE

WPLYW BIOSTYMULATORÓW NA ZAWARTOŚĆ ZWIĄZKÓW FENOLOWYCH W RÓŻACH BROKUŁU W CZASIE KRÓTKOTRWAŁEGO PRZECHOWYWANIA W TEMPERATURZE POKOJOWEJ

Abstract

Background. The world literature provides information about the effect of biostimulants on the biological value of broccoli immediately after harvesting, but there is little information how the quality of broccoli heads changes during storage in plants treated with biostimulants during cultivation. Therefore, the aim of this study was to evaluate the influence of an amino acid biostimulant and *Ascophyllum nodosum* filtrate on the content of phenolic compounds in broccoli heads – in fresh vegetables and in the ones stored for three days at room temperature.

Material and methods. The study was carried out on one broccoli cultivar: ‘Tiburón’. There were plants treated with the amino acid biostimulant, plants treated with *Ascophyllum nodosum* filtrate + amino acids, as well as control plants which were not treated with biostimulants. After harvesting the heads were stored at room temperature (24°C, 65% RH) for 1, 2 and 3 days. The contents of total phenols, caffeic, ferulic and sinapic acids, as well as the contents of quercetin and kaempferol were measured in fresh broccoli heads and after each day of storage.

Results. The biostimulants enhanced the total phenolic content and the amount of caffeic and ferulic acids in fresh broccoli heads only in the first year of the experiment. However, the content of kaempferol increased in both years of the experiment. The biostimulants considerably affected the total phenolic content, the amount of quercetin as well as the amounts of ferulic and sinapic acids after the second or third day of storage, but only in the first or second year of the experiment.

Conclusions. The biostimulants increased the content of phenolic compounds, but this effect was different in both years of the experiment. The total phenolic content and phenolic acids content increased with the length of storage.

Keywords: *Brassica oleracea* var. *italica*, amino acids, *Ascophyllum nodosum*

Introduction

Biostimulants are substances or microorganisms applied to horticultural and agricultural crops to increase yield, improve their quality, enhance plant tolerance to stress, and improve nutritional efficiency (du Jardin, 2015). Calvo et al. (2014) list five basic groups of biostimulants: microbiological inoculants, humic acids, fulvic acids, seaweed extracts, protein hydrolysates and amino acids.

Amino acids and peptide mixtures are obtained by chemical and enzymatic protein hydrolysis from agro-industrial by-products, both from plants (crop residues) and animal waste (e.g. collagen, epithelial tissues) (du Jardin, 2015). Some amino acid biostimulants also contain fats, macro- and microelements (Parrado et al., 2008). Protein hydrolysates stimulate carbon and nitrogen metabolism (Schiavon et al., 2008). They enhance plant defence responses and tolerance to stress including salinity, drought, temperature and oxidative stress (Apone et al., 2010; Ertani et al., 2013b).

Seaweed extracts stimulate the growth of the root system and increase the uptake of macro- and micronutrients from soil (Mancuso et al., 2006). Both amino acid biostimulants and seaweed extracts may increase crop yield (Fan et al., 2013; Zodape et al., 2011) and its biological value (Dobromilska and Gubarewicz, 2008). Biostimulants activate and stimulate the expression of the phenylalanine ammonialyase gene, the first enzyme of the phenylpropanoid pathway (Ertani et al., 2013a). As a result, plants are richer in phenolic compounds (Ertani et al., 2015). The correlations between the content of phenols and the use of biostimulants was confirmed in the studies by Fan et al. (2013) and Lola-Luz et al. (2013, 2014b). All these authors analysed the amount of phenolic compounds in fresh plants, but there was no information how the content of these compounds changed during storage. Therefore, the aim of this study was to evaluate the total phenolic content, the amounts of phenolic acids, quercetin and kaempferol not only in fresh heads treated with the amino acid biostimulant and amino acids + *Ascophyllum nodosum* filtrate, but also in plants stored at room temperature for a short time.

Material and methods

The experiment was conducted on one broccoli cultivar, ‘Tiburón’ in 2012 and 2013. The field trial was conducted in a randomised block design with four replications. The objects of the experiment were as follows: amino acid biostimulant (AA) – 1.5 dm³·ha⁻¹ (broccoli were sprayed 2, 4, and 6 weeks after planting), *Ascophyllum nodosum* filtrate (AN) – 1% (seedlings were watered 4 and 5 weeks after sowing) + AA – 1.5 dm³·ha⁻¹ (plants were sprayed 2, 4, and 6 weeks after planting), control plants (cultivated without biostimulants). The amino acid biostimulant used in the experiment contained 18 amino acids, both free form amino acids and short peptide chains. The

Ascophyllum nodosum filtrate was enriched with phosphorus (13% P₂O₅) and potassium (5% K₂O).

The transplants were planted in a field in mid-July. 35 plants at the stage of four leaves were planted in each plot of 8.75 m² and spaced at 0.5 × 0.5 m. The plants were cultivated without watering. In the first year of the experiment the broccoli heads were harvested on 11 September, whereas in the second year of the experiment they were harvested on 23 September. 24 evenly compact and sized heads were collected from each plot and put into storage. The experiment was conducted in a randomised block design with two replications (three heads in each replicate). Samples for analysis of the content of phenolic compounds were collected from fresh heads and after the first, second and third day of storage at room temperature (24°C, 65% RH). 50 g of broccoli heads was taken from each replication, broken and frozen at –20°C.

Phenolic compounds analysis

Frozen broccoli heads were homogenised in 80% methanol and then extracted at 40°C for 2 h. The total phenolic content was measured with the colorimetric method (Singleton and Rossi, 1965) with Folin-Ciocalteu reagent and gallic acid used as a standard. The absorbance was measured at $\lambda = 765$ nm using a Varian Cary 300 Bio UV-Visible spectrophotometer.

The concentration of phenolic compounds was determined after alkaline and acid hydrolysis using the method developed by Gliszczynska-Świągło et al. (2007). The broccoli samples were treated with 2M NaOH, boiled for 30 min, acidified, and then extracted with diethyl ether. Then, the samples were treated with 6M HCl, boiled for 30 min again, and extracted with diethyl ether. The combined extracts were dried and then they were re-dissolved in 1 ml of 80% ethanol injected into an HPLC column.

The HPLC analysis was made with a Waters Alliance 2695 Chromatograph coupled with a Waters 2996 Photodiode Array Detector using the method described above (Gliszczynska-Świągło et al., 2007). The chromatographic separation was carried out on an RP C-18 column, 250 × 4 mm × 5 μ m (at a temperature of 20°C). The mobile phase consisted of A (acetonitrile) and B (water and 2% acetic acid). The gradient elution was as follows: at the start: 100% B; 0–14 min: 90% B; 15–30 min: 90% B; 31–45 min: 82% B; 46–55 min: 100% A; 56–60 min: 100% A; 61–75 min: 100% B. The concentrations of phenolic acids were measured using an internal standard at wavelengths of $\lambda = 280$ nm (caffeic acid) or $\lambda = 320$ nm, and 280 nm (ferulic acid), whereas the wavelength of flavonoids was 320 nm. The compounds were identified by comparing the retention time of the peak under analysis with the retention time of the standard, and by adding a specific amount of the standard to the samples analysed and repeating the analysis. The detection level was 1 μ g/g. The phenolic acid and flavonoids, Folin-Ciocalteu's phenol reagent, NaOH, sodium carbonate and methanol were purchased from Sigma Aldrich (St. Louis, MO, USA). The diethyl ether was purchased from Honeywell (Germany).

Statistical analysis

The storage experiment was conducted in a two-factor design, with two replicates (three plants in each). The influence of the biostimulants and storage duration on the

content of phenolic compounds was determined with the *F*-test. Statistical analyses were carried out with the Stat program. The Duncan test at a significance level of $p < 0.05$ was used to estimate differences between the mean values.

Results and discussion

The analysis of variance showed that the biostimulants significantly affected the total phenolic content (Table 1). The total phenolic content in the fresh broccoli heads was higher only in the first year after the AA treatment (Table 2). The correlation between the amount of phenols and application of AA was confirmed by Nardi et al. (2016). According to Ertani et al. (2015), the use of protein hydrolysates resulted in a 40% increase in the total phenolic content in the leaves and red pepper fruit, whereas the content in green fruit increased by as much as 140%.

Table 1. ANOVA *F*-values for the total content of phenolics, phenolic acids and flavonols

| Variant | Total phenolics | Phenolic acids | | | Flavonols | |
|--------------------|-----------------|----------------|---------|---------|-----------|------------|
| | | caffeic | ferulic | sinapic | quercetin | kaempferol |
| 2012 | | | | | | |
| Day | ** | ** | ** | ** | ** | ** |
| Biostimulant | * | ** | * | ** | ns | ** |
| Day × biostimulant | ** | ** | ns | ** | * | * |
| 2013 | | | | | | |
| Day | ** | ** | ** | ** | ** | ** |
| Biostimulant | ** | ** | ** | * | ** | * |
| Day × biostimulant | ** | ** | ** | ns | ** | ** |

* – significance level $p < 0.1$, ** – significance level $p < 0.05$, ns – not significant at $p < 0.05$.

After three days of storage, the total phenolic content in the plants treated with AA and AA + AN was respectively 35% and 14% higher than the content in the control samples in the first year of the experiment. Fan et al. (2011, 2013) and Lola-Luz et al. (2014b) also reported an increase in the total phenolic content after the application of *Ascophyllum nodosum* extracts. However, these authors conducted their experiments on fresh plants without storage.

The biostimulants (both AA and AA + AN) used in our study increased the contents of caffeic, ferulic and sinapic acids, but their influence was not the same in both years of the experiment (Table 3). This correlation was also confirmed by Ertani et al. (2014), who observed that amino acids significantly increased the contents of caffeic and ferulic acids in pepper.

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Table 2. The content of total phenolics in broccoli heads during short-term storage (mg GAE per 100 g f. w.)

| Treatment | Day of storage | | | | Mean |
|-----------|----------------|---------|---------|---------|---------|
| | 0 | 1 | 2 | 3 | |
| | 2012 | | | | |
| Control | 2.74 c | 1.84 d | 3.19 bc | 3.76 a | 2.88 AB |
| AA + AN | 1.64 d | 1.89 d | 3.53 ab | 3.75 a | 2.70 B |
| AA | 3.43 ab | 1.96 d | 3.39 ab | 3.40 ab | 3.04 A |
| Mean | 2.60 C | 1.89 D | 3.37 B | 3.63 A | × |
| | 2013 | | | | |
| Control | 2.64 h | 4.57 fg | 5.39 d | 6.88 c | 4.87 B |
| AA + AN | 2.71 h | 5.06 de | 5.38 d | 7.87 b | 5.26 A |
| AA | 2.81 h | 4.86 ef | 4.36 g | 9.30 a | 5.33 A |
| Mean | 2.72 D | 4.83 C | 5.05 B | 8.01 A | × |

AA – amino acids, AN – *Ascophyllum nodosum* filtrate.

Values in each column in each year marked with the same small or with the same capital letter do not differ significantly at $p < 0.05$.

Values in each row in each year marked with the same capital letter do not differ significantly at $p < 0.05$.

Table 3. The content of phenolic acids in broccoli heads during short-term storage ($\mu\text{g/g}$ f.w.)

| Treatment | Day of storage | | | | Mean |
|-----------|----------------|---------|---------|---------|---------|
| | 0 | 1 | 2 | 3 | |
| 1 | 2 | 3 | 4 | 5 | 6 |
| | Caffeic acid | | | | |
| | 2012 | | | | |
| Control | 11.03 b | 7.18 g | 8.08 f | 9.97 cd | 9.07 B |
| AA + AN | 10.96 b | 7.50 g | 9.10 e | 10.29 c | 9.46 A |
| AA | 13.08 a | 7.33 g | 8.30 f | 9.70 d | 9.60 A |
| Mean | 11.69 A | 7.34 D | 8.49 C | 9.99 B | × |
| | 2013 | | | | |
| Control | 8.60 f | 7.99 g | 9.86 de | 14.27 c | 10.18 C |
| AA + AN | 7.41 h | 8.49 f | 10.17 d | 16.31 b | 10.59 B |
| AA | 7.25 h | 8.21 fg | 9.59 e | 18.77 a | 10.95 A |
| Mean | 7.75 D | 8.23 C | 9.87 B | 16.45 A | × |

Table 3 – cont.

| 1 | 2 | 3 | 4 | 5 | 6 |
|---------|--------------|-----------|-----------|-----------|----------|
| | Ferulic acid | | | | |
| | 2012 | | | | |
| Control | 7.00 de | 6.15 f | 8.14 bc | 14.74 a | 9.01 B |
| AA + AN | 7.50 cd | 6.40 ef | 8.34 b | 14.74 a | 9.24 B |
| AA | 8.51 b | 6.37 ef | 8.55 b | 15.10 a | 9.63 A |
| Mean | 7.67 C | 6.30 D | 8.34 B | 14.86 A | × |
| | 2013 | | | | |
| Control | 7.56 f | 10.64 e | 18.15 bc | 15.49 d | 12.96 C |
| AA + AN | 7.87 f | 10.88 e | 18.14 bc | 17.70 c | 13.65 B |
| AA | 7.84 f | 10.52 e | 18.59 b | 20.37 a | 14.33 A |
| Mean | 7.76 D | 10.68 C | 18.29 A | 17.85 B | × |
| | Sinapic acid | | | | |
| | 2012 | | | | |
| Control | 8.52 g | 12.46 f | 14.19 def | 15.97 bcd | 12.78 B |
| AA + AN | 8.66 g | 17.67 ab | 16.42 abc | 16.60 abc | 14.84 A |
| AA | 9.12 g | 13.95 ef | 15.45 cde | 18.05 a | 14.14 A |
| Mean | 8.77 C | 14.69 B | 15.35 B | 16.87 A | × |
| | 2013 | | | | |
| Control | 6.94 d | 11.56 c | 13.01 bc | 13.50 abc | 11.25 B |
| AA + AN | 7.06 d | 13.38 abc | 13.52 abc | 13.51 abc | 11.86 AB |
| AA | 7.43 d | 12.58 c | 14.71 ab | 15.18 a | 12.47 A |
| Mean | 7.14 C | 12.51 B | 13.74 A | 14.06 A | × |

AA – amino acids, AN – *Ascochyllum nodosum* filtrate.

Values in each column in each year marked with the same small or with the same capital letter do not differ significantly at $p < 0.05$.

Values in each row in each year marked with the same capital letter do not differ significantly at $p < 0.05$.

The total phenolic content and the amounts of ferulic, sinapic and caffeic acids increased along with the duration of storage both in the plants treated with the biostimulants and in the control plants. This is consistent with the results of our previous studies (Kałużewicz et al., 2016), where the total phenolic content increased by more than 180% after three days of storage, whereas the content of caffeic, ferulic and sinapic acids grew by 60%, 198% and 151%, respectively. Leja et al. (2001) and Starzyńska et al. (2003) observed the same trend in broccoli heads stored at room temperature. Kałużewicz et al. (2012) found that after 6 h of storage at 24°C there was a nearly 20% increase in the content of flavonoids.

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In our study the biostimulants increased the content of kaempferol in the fresh broccoli heads in both years of the experiment, but the amount of quercetin increased only in the second year (Table 4). This observation is consistent with the results of Lola-Luz et al. (2014a), who found a significant increase in the content of flavonols in onions and tomatoes after the application of *Ascophyllum nodosum* extract.

Table 4. The content of quercetin and kaempferol in broccoli heads during short-term storage ($\mu\text{g/g}$ f. w.)

| Treatment | Day of storage | | | | Mean |
|------------|----------------|-----------|-----------|-----------|---------|
| | 0 | 1 | 2 | 3 | |
| Quercetin | | | | | |
| 2012 | | | | | |
| Control | 13.82 a | 9.09 e | 10.38 d | 13.36 ab | 11.66 A |
| AA + AN | 12.45 bc | 10.34 d | 11.43 cd | 13.28 ab | 11.87 A |
| AA | 13.46 ab | 9.23 a | 10.99 d | 13.61 a | 11.82 A |
| Mean | 13.24 A | 9.55 C | 10.93 B | 13.42 A | × |
| 2013 | | | | | |
| Control | 11.30 d | 12.90 bc | 16.61 a | 11.03 d | 12.96 C |
| AA + AN | 12.85 bc | 14.20 b | 16.51 a | 14.16 b | 14.43 A |
| AA | 11.48 cd | 13.67 b | 13.55 b | 16.15 a | 13.71 B |
| Mean | 11.87 C | 13.59 B | 15.55 A | 13.78 B | × |
| Kaempferol | | | | | |
| 2012 | | | | | |
| Control | 58.26 b | 50.21 g | 51.61 ef | 57.75 b | 54.45 B |
| AA + AN | 57.44 bc | 52.05 efg | 55.59 bcd | 54.72 cde | 54.95 B |
| AA | 61.50 a | 53.99 def | 55.32 bcd | 57.48 bc | 57.07 A |
| Mean | 59.07 A | 52.08 D | 54.17 C | 56.65 B | × |
| 2013 | | | | | |
| Control | 56.24 f | 57.81 def | 64.69 ab | 68.03 a | 61.69 A |
| AA + AN | 58.31 def | 62.27 bc | 61.29 bcd | 57.40 ef | 59.81 B |
| AA | 60.49 cde | 61.97 bc | 64.39 b | 62.01 bc | 62.21 A |
| Mean | 58.34 C | 60.68 B | 63.46 A | 62.48 AB | × |

AA – amino acids, AN – *Ascophyllum nodosum* filtrate.

Values in each column in each year marked with the same small or with the same capital letter do not differ significantly at $p < 0.05$.

Values in each row in each year marked with the same capital letter do not differ significantly at $p < 0.05$.

Many authors report that kaempferol is the major flavonoid found in the broccoli plant (Gliszczyńska-Świgło et al., 2007; Kałużewicz et al., 2012; Koh et al., 2009). According to Hollman and Arts (2000), the content of kaempferol in broccoli heads is twice as high as the content of quercetin. This tendency was also observed in our study, where the average content of kaempferol in the fresh heads was almost six times greater than the content of quercetin in both years of the research. In the second year of the research the content of both flavonols was higher than in the first year. The content of quercetin increased by 24%, while the content of kaempferol increased by 10%. Horbowicz and Babik (2005) also reported considerable differences in the amounts of both flavonols in the first and second year of their study. According to the authors, the content of quercetin differed by about 20%, whereas the content of kaempferol differed by about 27%. The authors also observed that the cultivars significantly differed in the content of these flavonols, even as much as two–three times.

In the second year of our study both the content of quercetin and kaempferol increased with the length of storage. However, this trend was not observed in the first year of the experiment, when the content of quercetin after three days of storage was the same as in the fresh heads, while the content of kaempferol decreased. The increase in the contents of quercetin and kaempferol during storage in the second year is consistent with the results of another study by Kałużewicz et al. (2016). The researchers observed that after three days of storage at a temperature of 21°C the amount of quercetin increased by 56%, while the content of kaempferol grew by 27%.

The differences in the effectiveness of the biostimulants, which depended on the content of phenolic compounds, can be explained by different weather conditions during the research. In 2012 during the growth period the total rainfall was twice lower than in 2013 (Table 5). In addition, there were very big differences in temperatures between the years of the research, especially during the 30 days preceding the harvest. The influence of growing conditions on the content of phenolic compounds was also observed by Gliszczyńska-Świgło et al. (2007) and Vallejo et al. (2003).

Table 5. Temperature and total rainfall during broccoli cultivation

| Year | Temperature (°C) | | | Total rainfall (mm) |
|------|--------------------------|---------|-------|---------------------|
| | maximum | minimum | daily | |
| | From planting to harvest | | | |
| 2012 | 24.0 | 13.1 | 18.3 | 68.0 |
| 2013 | 21.2 | 11.0 | 16.1 | 132.4 |
| | 30 days before harvest | | | |
| 2012 | 24.0 | 12.6 | 18.3 | 19.0 |
| 2013 | 14.7 | 6.2 | 10.4 | 46.4 |

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Conclusions

1. The biostimulants increased the content of phenolic compounds, but this effect was different in both years of the research.

2. The total phenolic content and the amount of phenolic acids increased with the length of storage both in the control samples and in the plants treated with the biostimulants. This trend was not observed in quercetin and kaempferol.

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WPLYW BIOSTYMULATORÓW NA ZAWARTOŚĆ ZWIĄZKÓW FENOLOWYCH W RÓŻACH BROKUŁU W CZASIE KRÓTKOTRWAŁEGO PRZECHOWYWANIA W TEMPERATURZE POKOJOWEJ

Abstrakt

Wstęp. W światowej literaturze można znaleźć informacje dotyczące wpływu biostymulatorów na wartość biologiczną brokułu zaraz po zbiorze róż, nie ma natomiast informacji dotyczących tego, jak zmienia się jakość róż w czasie przechowywania tych roślin, które w okresie uprawy były traktowane biostymulatorami. Celem badań była ocena wpływu biostymulatorów zawierających aminokwasy oraz filtrat *Ascophyllum nodosum* na zawartość związków fenolowych w świeżych różach brokułu oraz podczas 3-dniowego przechowywania w temperaturze pokojowej.

Material i metody. Badania obejmowały jedną odmianę brokułu: ‘Tiburon’. Zastosowano następujące obiekty: rośliny kontrolne (niektrowane biostymulatorami), rośliny traktowane biostymulatorami zawierającymi aminokwasy, rośliny traktowane biostymulatorami zawierającymi aminokwasy + filtrat *Ascophyllum nodosum*. Zebrane różę były przechowywane przez 1, 2 i 3 dni w temperaturze pokojowej (24°C) i wilgotności względnej powietrza 65%. Po zbiorze oraz po kolejnych dniach przechowywania oznaczono zawartość fenoli ogółem, kwasu kawowego, ferulowego, synapinowego oraz kwercetyny i kemferolu.

Wyniki. Biostymulatory wpłynęły na zwiększenie zawartości fenoli ogółem, kwasu kawowego i kwasu ferulowego w świeżych różach brokułu w pierwszym roku badań oraz kemferolu w obu latach badań. W przypadku zawartości fenoli ogółem, kwasu ferulowego, synapinowego i kwercetyny wpływ biostymulatorów stwierdzono po drugim lub po trzecim dniu przechowywania w jednym tylko roku badań (w pierwszym lub w drugim).

Wnioski. Biostymulatory wpłynęły na zwiększenie zawartości badanych związków fenolowych, lecz efekt ten był inny w każdym roku. Zawartość fenoli ogółem oraz kwasów fenolowych zwiększała się wraz z długością okresu przechowywania.

Słowa kluczowe: *Brassica oleracea* var. *italica*, aminokwasy, *Ascophyllum nodosum*

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