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Brassinosteroid improves content of antioxidants in seeds of selected leguminous plants

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Abstract

The content of antioxidants (tocopherols, trocotrienols, ascorbic acid, β-carotene) as well as soluble proteins, total fats and soluble sugars were studied in the seeds of pea and lupine after application of brassinosteroid (24-epibrassinolide). Plants were cultured in separate pots in an open vegetation hall in 2010. The following year, a field experiment was carried out in a randomized block design where plants were growing in rows, directly in the soil. 24-Epibrassinolide (0.25-0.5 mg dm^{-3}) was applied via spraying or watering of the flowering plants. Experiments were done during natural vegetation seasons (spring-summer, latitude: 50°03' N, longitude: 19°55' E) and seeds were collected after maturation. In the pea pot experiment, γ-tocopherol content was increased (9%-15%) in seeds after brassinosteroid spraying (0.5 mg dm^{-3}). In the lupine field experiment, α- and γ-tocopherol content was elevated in seeds (8%-84%) after brassinosteroid application (0.25 mg dm^{-3}) via both methods. In the pea field and pot experiment, the ascorbic acid content was increased (18-52%) after watering plants with 24-epibrassinolide (0.25 mg dm^{-3}). In lupine seeds, ascorbic acid as well as β-carotene content was elevated after brassinosteroid watering (0.25 mg dm^{-3}). The brassinosteroid effect on the content of proteins, sugars, and fats in seeds was also studied. The results obtained in our study show the possibility of using brassinosteroid to manipulate plant seed content, which may be important from a nutritional point of view. Moreover, the studies reveal some mechanisms of brassinosteroid action on the metabolism of seeds.

Keywords: ascorbic acid; β-carotene; 24-epibrassinolide; food quality; lupine; pea; tocopherols.
Abbreviations: BRs_brassinosteroids, BR_brassinosteroid, BR_{27}_24-epibrassinolide (according to Zullo and Kohout, 2004), ABA_ abscisic acid.

Introduction

Legumes, including peas, beans and lupines, are among the most important staple food crops worldwide (Amarteifio and Moholo, 1998). Legumes are also sown as an underplant crop because of their ability to enrich soil nitrogen content. Legumes have been also recommended for people from risk groups to increase the consumption of proteins, minerals and vitamins, which are important dietary components and a valuable basic material for the food and animal feed industries (Adewusi and Falade, 1996). Nutritionally, legumes are particularly good sources of proteins, carbohydrates, minerals, vitamins, and fiber. They are especially important as protein sources in areas where animal-derived proteins are scarce. Legumes contribute about 18% of the whole plant-derived proteins consumed by humans (Duranti, 2006). Additionally, legume proteins contain large amounts of some essential amino acids, such as lysine, but are low in amino acids containing sulphur such as methionine and cysteine. Legume carbohydrate content varies from 13% to 65%, of which half or more is starch. Many legumes are also good sources of iron, calcium, phosphorus, zinc, copper and magnesium (Amarteifio and Moholo, 1998). They are particularly enriched in B vitamins, important for human nutrition, such as thiamin, riboflavin, niacin, folic acid and pantothenic acid, as well as vitamin C. Legumes are also valuable sources of tocopherols (Boschin and Arnoldi, 2011). The chemical composition of legume seeds largely depends on the cultivar and environmental factors during growth such as temperature, soil type, fertilization and the occurrence of stress factors etc. (Gül et al., 2008; Vlahakis and Hazebroek, 2000). Changes in the chemical composition of the seeds can also be induced by so-called plant growth regulators. Plant growth regulators, including plant hormones, are organic substances, which in small amounts can modify the physiological functions of the plant. This modification is based on the enhancement or inhibition of growth and developmental processes such as, e.g. germination, root formation, fruit setting and aging of plants and the improvement of a plant's stress resistance. Modification of these essential processes can help to increase the quality and quantity of plants' yield. In agriculture, horticulture and arboriculture, synthetic regulators are used (inhibitors of the biosynthesis of gibberellins – CCC), although natural hormones are also applied: mainly ethylene for banana ripening or auxins and cytokinins for obtaining partenocarpic fruits.

In past decades there has been an increasing interest in the practical use of relatively new plant steroid hormones, i.e. brassinosteroids (Hayat and Ahmad, 2010). Brassinosteroids (BRs) comprise about 70 compounds that show structural similarities with steroid hormones of animals, insects, and humans (Bajguz, 2007). Studies on brassinosteroid insensitive mutants as well as experiments with BR inhibitors and genetic studies have provided compelling evidence that BRs are essential for plant growth and development

(Kauschmann et al., 1996; Li et al., 1996; Szekeres et al., 1996). Several studies with external application of these hormones have demonstrated that BRs influenced plant growth, seed germination, nitrogen fixation, senescence, leaf abscission, and enhanced tolerance against cold stress, salt stress and diseases (Clouse and Sasse, 1998; Nakashita et al., 2003; Bajguz and Hayat 2009). Several practical applications of BRs in agriculture have been reported (Hayat and Ahmad, 2010). The application of brassinosteroids to legume plants usually aims at improving stress resistance and yield (Ramraj et al., 1997; Vardhini and Rao, 1998; Hasan et al., 2008). Considerably fewer studies are devoted to the influence of BRs on the quality of the yield of legumes (Vardhini and Rao, 1998; Janeczko et al., 2009; Janeczko et al., 2011).

For many years there has been a great interest in antioxidants due to their beneficial effects on human health as free radical scavengers and their ability for food preservation. Antioxidants are molecules with a low reduction potential that can donate electrons or hydrogen atoms and thereby prevent the oxidation of other molecules (Asensi-Fabado and Munné-Bosch, 2010). The most popular natural antioxidants include tocopherols, ascorbic acid, and carotenoids. The content of antioxidants in food may be increased by their addition to food products but also by methods of metabolomic engineering. However, other methods of increasing the content of antioxidants in plant foods should not be neglected. The use of natural environmentally friendly plant growth regulators could be useful in so-called ecological agriculture.

The aim of our study was to evaluate the effect of brassinosteroid (24-epibrassinolide) on the chemical content of seeds of leguminous plants. The content of antioxidants (tocopherols, trocotrienols, ascorbic acid, β-carotene) was the main interest, but we also assayed soluble proteins, total fats, and soluble sugars in the seeds of *Pisum sativum* L. and *Lupinus luteus* L. treated with 24-epibrassinolide *via* spraying or watering of plants at the flowering stage.

Results

The content of individual seed components obviously differed for different species (pea and lupine) and cultivars. The composition of the seeds was also influenced by growth conditions (field and pot experiments) and the method of treatment (spraying, watering). Hormonal treatment clearly modified the composition of newly formed seeds, although this effect was partly dependent on the species, the method of application and growth conditions.

The influence of BR_{27} on the yield quality of pea

Significant changes have been noted in the concentration of tocopherols after 24-epibrassinolide (BR_{27}) application in the pot experiment. Higher content of α-tocopherol was reported after BR-spraying plants of Wiato cultivar (Table 1). An increase in γ-tocopherols occurred in both cultivars, Roch (9%) and Wiato (15%), sprayed with BR_{27}. No effect was observed on the δ-tocopherol level. In the field experiment, a similar effect to the pot cultures was found with respect to the α-tocopherol and γ-tocopherol levels. Additionally, the content of δ-tocopherol was increased by a few percent after watering plants of both cultivars with BR_{27}. Spraying of plants with BR_{27} increased the content of δ-tocopherol in cv. Wiato (6%), while only a slight tendency was noted in cv. Roch. The impact of brassinosteroid on tocotrienols was much weaker in comparison to tocopherols. 24-Epibrassinolide increased the content of γ-tocotrienol (6%) and δ-tocotrienol (27%) in the field experiment after watering cv. Roch plants and after spraying cv. Wiato plants.

Higher concentrations of β-carotene by about 50% were observed only after spraying cv. Roch plants (field experiment, Fig. 1) and watering cv. Wiato plants (pot experiment; Fig. 1).

BR_{27} increased the ascorbic acid content in seeds of both cultivars in the pot and field experiments (Fig. 1.). The most stable method of application was plant watering with BR_{27}. The effect of BR in this case was found for both cultivars in the pot and field experiments. The content of ascorbic acid in seeds was increased from 18% to more than 50%. Plants sprayed with BR_{27} increased the content of ascorbic acid from 10% to 30% in the seeds of cv. Roch and cv. Wiato (pot experiment) and cv. Roch (field experiment).

In the pot and field experiment, an increase in the protein content of about 11% to 19% was found in seeds of cv. Roch and cv. Wiato after watering plants with BR_{27} (Table 2). Surprisingly, a decrease of several percent in soluble proteins content was found in seeds of both cultivars in the field experiment and in the seeds of cv. Roch in the pot experiment after BR_{27} spraying.

Generally, an increase in the fat content (7%-37%) was noted in seeds of both cultivars in the pot and field experiment after BR_{27} spraying (Table 2). However, in case of cv. Roch cultured in pots, only a statistically insignificant tendency was detected. Watering plants with BR_{27} was effective in the case of cv. Wiato cultured in the field, where the total fat content was increased by about 11%. For cv. Roch, a tendency to increase total fats in the seeds was noted in plants cultured in the field. A similar trend was observed in case of cv. Wiato cultured in pots.

An increase in the concentration of soluble sugars (11%-18%) was obtained only for cv. Wiato cultured in pots after both methods of 24-epibrassinolide application (Table 2).

The influence of BR_{27} on the yield quality of lupine

Similarly to pea plants, exogenously applied BR_{27} changed the composition of the lupine seeds. In the pot experiment there was no effect of BR_{27} on α-tocopherol content in lupine seeds (Table 3). In the pot experiment only, cv. Talar reacted to brassinosteroid by an increased amount of γ-tocopherol (22%-30%) in the seeds. Regardless of the method of application used, the content of δ-tocopherol in the seeds was increased for both cultivars (Table 3). However, the results were statistically significant only in the case of cv. Talar. In the case of cv. Mister, only a tendency to increase the amount of this compound was noted. In the field experiment, the content of α- and γ-tocopherol was increased by 24-epibrassinolide application in both cultivars no matter which method of application was used (Table 3). The concentration of these two tocopherols was higher by 8% to 19% in the seeds of cv. Talar, while in seeds of cv. Mister it ranged from 11% to even 84%. The concentration of δ-tocopherol in seeds was increased only after BR_{27} watering (12%-20%). The content of seeds' tocotrienols after BR_{27} application remained unchanged.

The application of brassinosteroid also resulted in an increased concentration of β-carotene and ascorbic acid in lupine plants (Fig. 2). In the pot experiment (cv. Mister), the content of β-carotene was increased in seeds after plant spraying (13%) and plant watering (18%). A similar tendency was noted for cv. Talar, although it was statistically insignificant.

Table 1. The impact of 24-epibrassinolide on tocopherol and tocotrienol content in pea seeds. Mean values [µg g seeds $^{-1}$] followed by the same letter showed no significant differences (P<0.05) according to a Student's test (in columns, separately for each cultivar and method of BR_{27} application).

			POT EXPERIMENT			FIELD EXPERIMENT					
Cultivar	Application	BR_{27} [mg dm^{-3}]	α-tocopherol	γ-tocopherol	δ-tocopherol	α-tocopherol	γ-tocopherol	δ-tocopherol	α-tocotrienol	γ-tocotrienol	δ-tocotrienol
Roch	Spraying of plants	0.0	0.88a	66.0b	0.96a	0.48a	41.0b	7.02a	0.60a	2.96a	0.19a
		0.5	0.89a	72.2a	0.94a	0.58a	48.4a	7.08a	0.61a	3.09a	0.18a
	Watering of plants	0.0	0.49a	66.9a	0.98a	0.59a	48.8a	6.77b	0.62a	1.22a	0.18b
		0.25	0.57a	62.9a	0.98a	0.56a	48.2a	7.18a	0.63a	1.18a	0.23a
Wiato	Spraying of plants	0.0	0.73b	57.5b	0.95a	0.61b	15.7b	7.05b	0.62a	2.96b	0.18a
		0.5	0.96a	66.0a	0.98a	0.91a	35.7a	7.45a	0.66a	3.14a	0.19a
	Watering of plants	0.0	0.90a	55.0a	0.94a	0.69a	11.5a	7.21b	0.62a	2.92a	0.19a
		0.25	1.00a	55.0a	0.98a	0.67a	11.9a	7.51a	0.61a	2.85a	0.18a

Table 2. The impact of 24-epibrassinolide on soluble protein, total fat and soluble sugar content in pea seeds. Mean values [mg g seeds $^{-1}$] followed by the same letter showed no significant differences (P<0.05) according to a Student's test (in columns, separately for each variety and method of BR_{27} application).

			POT EXPERIMENT			FIELD EXPERIMENT		
Cultivar	Application	BR_{27} [mg dm^{-3}]	Soluble proteins	Total fats	Soluble sugars	Soluble proteins	Total fats	Soluble sugars
Roch	Spraying of plants	0.0	330.1a	98.9a	108.1a	362.9a	113.2b	71.0a
		0.5	285.9b	105.5a	100.9a	341.8b	154.9a	70.5a
	Watering of plants	0.0	306.9b	119.8a	122.1a	324.3b	126.2a	78.1a
		0.25	344.5a	113.6a	112.8a	360.9a	128.3a	77.5a
Wiato	Spraying of plants	0.0	323.7a	84.7b	105.3b	277.8a	119.0b	99.8a
		0.5	328.8a	110.9a	124.0a	268.6b	127.8a	93.5a
	Watering of plants	0.0	284.0b	85.6a	121.1b	282.1b	124.1b	108.5a
		0.25	320.9a	94.3a	134.8a	335.5a	137.9a	110.0a

Table 3. The impact of 24-epibrassinolide on tocopherol and tocotrienol content in lupine seeds. Mean values [µg g seeds $^{-1}$] marked by the same letter showed no significant differences (P<0.05) according to a Student's test (in columns, separately for each variety and method of BR_{27} application).

			POT EXPERIMENT			FIELD EXPERIMENT					
Cultivar	Application	BR_{27} [mg dm^{-3}]	α-tocopherol	γ-tocopherol	δ-tocopherol	α-tocopherol	γ-tocopherol	δ-tocopherol	α-tocotrienol	γ-tocotrienol	δ-tocotrienol
Talar	Spraying of plants	0.0	0.98a	66.5b	1.17b	1.98b	57.5b	5.77a	0.61a	2.98a	0.21a
		0.25	1.00a	80.9a	1.94a	2.16a	62.1a	5.72a	0.62a	2.95a	0.21a
	Watering of plants	0.0	0.97a	60.1b	1.43b	1.94b	55.7b	5.72b	0.62a	2.91a	0.21a
		0.25	0.98a	78.4a	2.24a	2.30a	62.0a	6.40a	0.62a	2.92a	0.21a
Mister	Spraying of plants	0.0	0.97a	79.9a	1.33a	1.17b	47.1b	5.49a	0.62a	2.72a	0.18a
		0.25	0.98a	79.2a	1.89a	2.15a	56.0a	5.50a	0.62a	2.78a	0.19a
	Watering of plants	0.0	0.98a	68.3a	1.42a	1.45b	51.4b	5.41b	0.62a	2.60a	0.18a
		0.25	0.96a	66.4a	1.72a	2.08a	56.8a	6.50a	0.62a	2.70a	0.19a

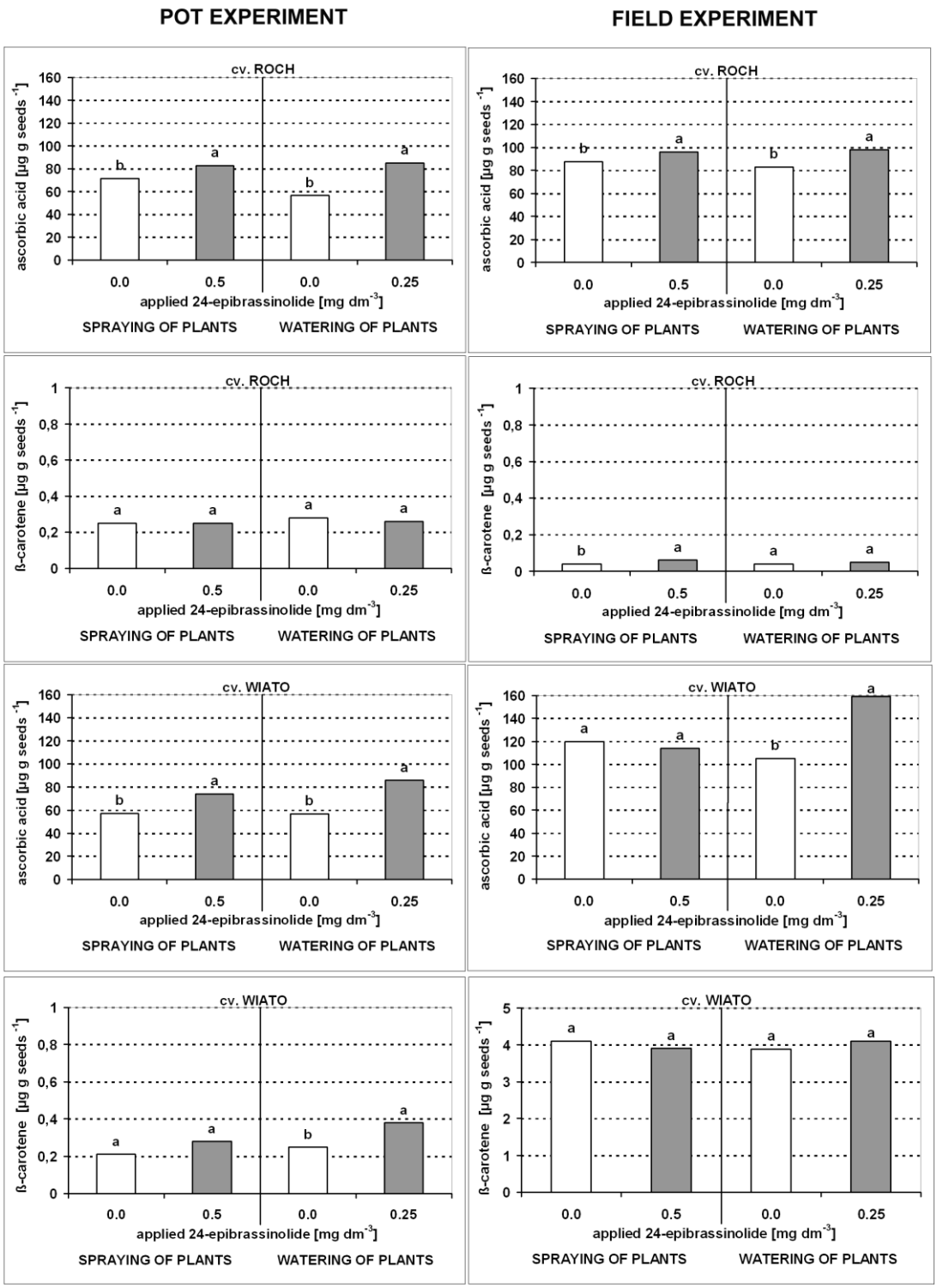

Fig 1. The impact of 24-epibrassinolide on the content of ascorbic acid and β-carotene in seeds of two pea cultivars (Roch and Wiato). Mean values [µg g seeds $^{-1}$] followed by the same letter showed no significant differences ($P<0.05$) according to a Student's test; comparison in pairs: 24-epibrassinolide treatment – control, separately for each variety and method of application.

In the field conditions, the β-carotene content in the seeds was increased after plant spraying (cv. Talar) and plant watering (cv. Mister). In the pot experiment, the content of ascorbic acid was increased after plant spraying (46%) and plant watering (40%) in case of seeds of cv. Mister. In the field conditions, the ascorbic acid content of the seeds was increased by a third after plant spraying (cv. Talar) as well as plant watering (cv. Mister).

An increase in the proteins, sugars, and fats was detected in lupine seeds after watering, as well as spraying the plants with brassinosteroid. The content of soluble proteins of the seeds increased after plant spraying with BR_{27} in the pot experiment (cv. Talar and cv. Mister) and in the field experiment (cv. Mister). The protein content increased by about 53% to 63%

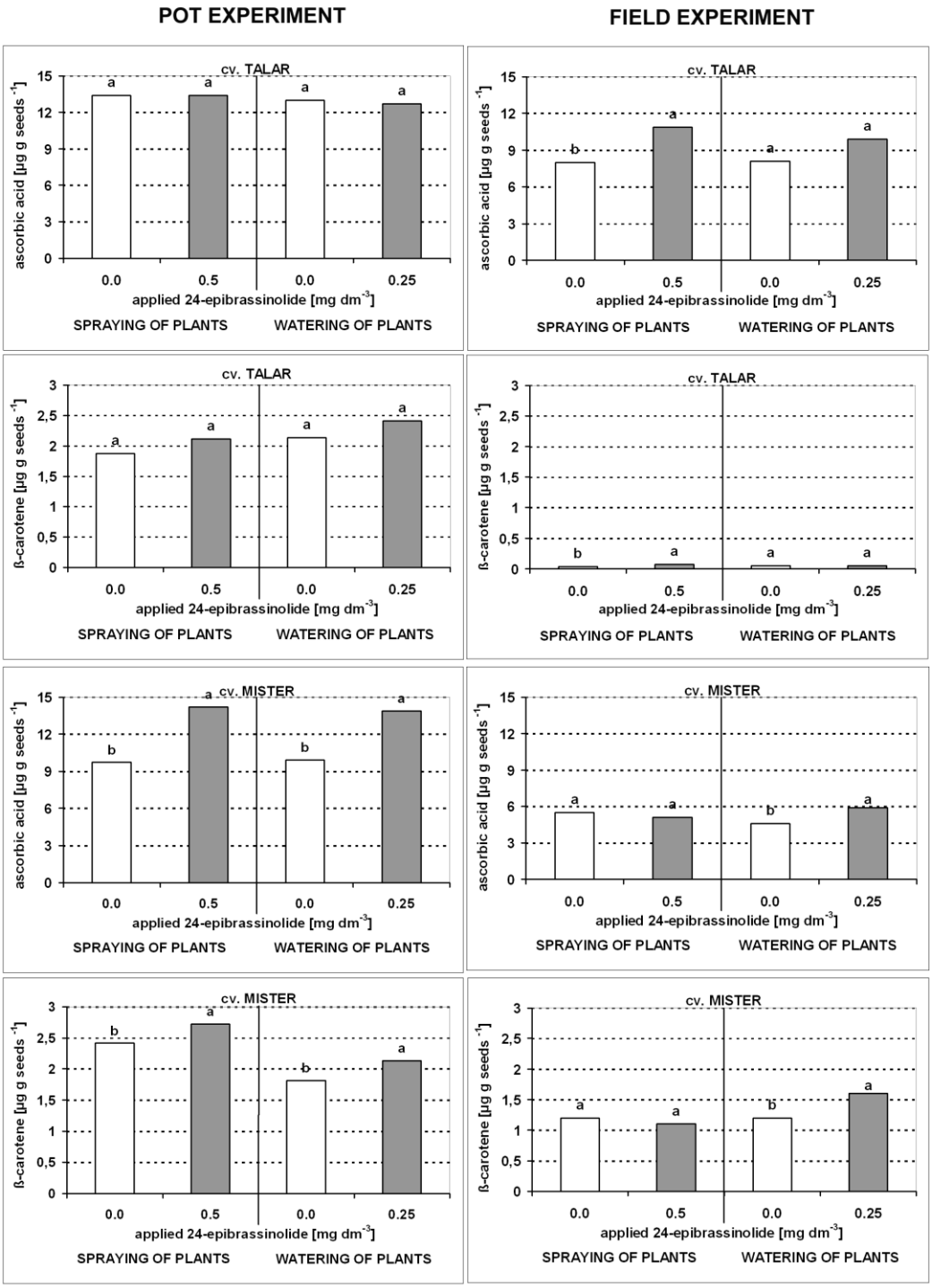

Fig 2. The impact of 24-epibrassinolide on the content of ascorbic acid and β-carotene in seeds of two lupine cultivars (Talar and Mister). Mean values [µg g seeds $^{-1}$] followed by the same letter showed no significant differences ($P<0.05$) according to a Student's test; comparison in pairs: 24-epibrassinolide treatment – control, separately for each variety and method of application.

(Table 4). Plant watering with BR_{27} increased the soluble proteins' content of the seeds of cv. Mister (field experiment) and cv. Talar (pot experiment). BR_{27} only affected the total fat content of cv. Talar in the pot and field experiments (fat increase in range of 33%-65%), while cv. Mister was not responsive to BR_{27} action (Table 4). The concentration of seed sugars was higher in BR_{27} watered plants of cv. Talar (pot and field culture) and cv. Mister (pot culture) by about 13%, 12% and 41%, respectively (Table 4). Spraying with BR increased the amount of soluble sugars in seeds of cv. Talar by about 52% (pot experiment) and cv. Mister by about 9% (field experiment).

Discussion

Brassinosteroid effect on the content of non-enzymatic antioxidants in seeds of legume plants

Literature data provides little information about the effect of brassinosteroids on the yield of legume plants (Ramraj et al., 1997; Vardhini and Rao, 1998; Fariduddin et al., 2004; Hasan et al., 2008; Janeczko et al., 2011). In most cases, where the positive effect of BRs were found on crop yield, the quality of it has not been studied. Most often, the yield quality of legumes was analyzed after BR application in the experiments performed on groundnut and soybean (Vardhini and Rao, 1998; Janeczko et al., 2009; Janeczko et al., 2011). Data obtained in this study show that hormonal treatment clearly modifies the composition of newly formed seeds. However, this effect partly depends on the species, cultivar, method of application, and the component tested. Nevertheless, the pot experiment and field cultivation proved that BR_{27} influences the composition of seeds acquired. This study focused in particular on the impact of BR on the content of nonenzymatic antioxidants such as tocopherols, tocotrienols, ascorbic acid, and β-carotene. Numerous studies indicate that BRs – as plant steroid hormones with anti-stress properties – stimulate the activity of antioxidant enzymes (Mazorra et al., 2002; Hasan et al., 2008). Less is known about the impact of BRs on nonenzymatic antioxidants such as ascorbic acid, tocopherols, and β-carotene.

Tocopherols (especially α-tocopherol) in plants are primarily associated with the response of plants to stress as they form together with ascorbate and glutathione, the so-called triad, actively counteracting the excessive growth of reactive oxygen species (Szarka et al., 2012). Tocotrienols have a similar role and participate in scavenging lipid peroxy radicals thus helping to maintain membrane integrity (Munné-Bosch and Alegre, 2002). Currently, it is known that the synthesis of tocopherols in plants is regulated by stress hormones, i.e., abscisic acid (ABA), salicylic acid, and jasmonic acid (Szarka et al., 2012). Our present results, together with earlier findings (Janeczko et al., 2009), show that BRs are the next group of hormones capable of regulating the production of tocochromanols in plants. Hence, the unanswered question remains whether BRs are influencing the production of tocochromanols directly, or rather indirectly by increasing the production of salicylic acid and other aforementioned stress hormones, which in turn stimulate the production of tocochromanols.

Ascorbic acid is involved in plants in the regulation of photosynthesis, cell division, and expansion (Smirnoff 1996). It is also a part of a plant's defense against oxidative stress (Smirnoff 1996). Beside its antioxidant properties, recent research has revealed another role of ascorbic acid; that is, a substrate for many 2-oxoacid-dependent dioxygenases (Prescott and John, 1996; Pastori et al., 2003). Dioxygenases are involved in the synthesis of plant hormones such as ethylene, gibberelins and ABA, which indicates a possible link between ascorbic acid and plant hormonal management (De Tullio and Arrigoni, 2003). BR increased the ascorbic acid content in the leaves of *Raphanus sativus* L. in response to Cr (VI) stress (Choudhary et al., 2011). After BR treatment of tomato roots at the seedling stage, the developed fruits possessed a lower quantity of ascorbic acid (Ali et al., 2006). The study performed by Vardhini and Rao (2002) revealed that the administration of 28-homobrassinolide and 24-epibrassinolide to pericarp discs of tomato decreased the levels of ascorbic acid. We showed for the first time that BRs may also increase the content of this compound in the seeds, and this effect is observed in both tested species of the *Fabaceae* family. The role of ascorbic acid in mediating the antagonism between ABA and gibberellins during seed germination is well known (Ye and Zhang, 2012). Brassinosteroid as a regulator of ascorbic acid production can then be an important player in the complex network connected with hormone management and plant germination.

In plants, carotenoids (including β-carotene) are essential components required for photosynthesis, photoprotection, the production of carotenoid-derived phytohormones and flower coloration (Bartley and Scolnik, 1995; Cazzonelli, 2011). According to Ali et al. (2006), after BR treatment of tomato roots at the seedling stage, the fruit at ripening had higher levels of lycopene and β-carotene. In our previous work, an increased amount of β-carotene was found in seeds of soybean and oilseed rape after application of 24-epibrassinolide (Janeczko et al., 2009). Currently, the best described are the positive and negative metabolite feedback mechanisms existing within the carotenoid pathway and among carotenoids and hormone ABA (Cazzonelli, 2011). Apparently, plant steroid hormones are also involved in carotenoid metabolism. Theoretically, BRs may have a direct effect on the activity of enzymes involved in pathways of carotenoid biosynthesis. The indirect effect of BRs i.e. *via* regulation of ABA synthesis is also possible, which in turn regulates carotenoid production.

Brassinosteroid effect on content of proteins, sugars, and fats in seeds of legume plants

Numerous studies on the impact of BRs on other parameters of the qualitative composition of leguminous seeds (protein, fats and sugars content) correlate with the results obtained in our work. Numerous studies have shown that BRs stimulate protein synthesis in the leaves of plants growing under both control conditions and those treated with stressors

(Anuradha and Rao, 2001; Sirhindi, 2009). An increase in the content of soluble proteins was also found in seeds of *Arachis hypogea* L. after brassinolide and 24-epibrassinolide spraying (Vardhini and Rao, 1998). However, this phenomenon was not observed in soybean seeds (Janeczko et al., 2011). The increase in protein content under brassinosteroid influence is a result of the enhanced activity of RNA and DNA polymerases that are engaged in a physiological response to the BR hormone (Kalinich et al., 1986). Present studies generally show an increase in protein content after BR application to pea and lupine plants. However, it should be noted that the Bradford method applied, which only allowed measuring of the soluble proteins. Therefore, it is not known to what extent BR modified the content of total the proteins. This issue should be further investigated, and positive results would open up the possibility of utilizing BRs in improving the protein content in the seeds of selected legumes.

The positive impact of the BRs on the production and metabolism of carbohydrates is quite well known in plants (Yu et al., 2004; Vardhini et al., 2011). The results presented in this study also indicate an increase in the sugar content in seeds of pea and lupine obtained after BR_{27} treatment. These results correspond with a study performed by Vardhini and Rao (2002) that revealed the ability of BR to accelerate tomato fruit maturation, correlated with the increased content of carbohydrates. This stimulation of the production of carbohydrates might be caused by an enhanced photosynthe-

Table 4. The impact of 24-epibrassinolide on soluble protein, total fat and soluble sugar content in lupine seeds. Mean values [mg g seeds $^{-1}$] followed by the same letter showed no significant differences ($P<0.05$) according to a Student's test (in columns, separately for each variety and method of BR_{27} application).

			POT EXPERIMENT			FIELD EXPERIMENT		
Cultivar	Application	BR_{27} [mg dm^{-3}]	Soluble proteins	Total fats	Soluble sugars	Soluble proteins	Total fats	Soluble sugars
Talar	Spraying of plants	0.0	176.8b	216.5b	118.2b	259.9a	141.1a	61.4a
		0.25	287.3a	291.7a	179.1a	261.5a	149.9a	68.8a
	Watering of plants	0.0	102.8b	100.6b	143.1b	285.8a	118.0b	67.6b
		0.25	155.9a	164.5a	161.9a	284.9a	156.9a	75.9a
Mister	Spraying of plants	0.0	147.8b	138.4a	128.1a	115.9b	190.1a	106.4b
		0.25	225.6a	129.5a	135.9a	186.2a	188.8a	116.1a
	Watering of plants	0.0	153.8a	159.2a	128.1b	298.8b	144.0a	104.7a
		0.25	155.9a	162.9a	180.1a	341.4a	120.0a	101.4a

tic capacity of plants under the influence of brassinosteroids. In fact, an increase in CO_2 fixation and levels of reducing sugars was also reported in wheat and mustard plants after the application of brassinolide (Braun and Wild, 1984). In addition to the effect of BR on CO_2 fixation, it has also been found that these compounds regulate the distribution of assimilates (Fuji and Saka, 1992; 2001). In rice, brassinolide decreased the starch content in leaf sheaths and culms, and increased the content of starch and sucrose in hulled grains.

In comparison to studies on protein and sugars, not much is known about the effect of BR on fats in plants. In *Arachis hypogaea* L., exogenous application of brassinolide and 24-epibrassinolide by foliar spray resulted in an elevated fat content of seeds of up to 30% (Vardhini and Rao, 1998). Janeczko et al. (2009) observed only slight changes in molar percentage of particular fatty acids in soybean, wheat, and oilseed rape seeds after application of 24-epibrassinolide. Current studies reveal an increased amount of total fats in both cultivars of pea and one cultivar of lupine cultivated in the field and pot experiments. The content of total fats was generally increased by about 30%. However there were extreme cases where the amount of fats was increased by about 60%, while sometimes the increase was only a few percent. The mechanism of BR action on lipids' metabolism requires further investigation.

General comments

BR-regulated plant response to environmental conditions is the result of a complex sequence of biochemical reactions such as activation or suppression of key enzymatic reactions, induction protein synthesis and the production of various defensive chemical compounds (Bajguz and Hayat, 2009). Our results supplement existing knowledge on the BR method of action. We show, that the response of plants to exogenous BR treatment is reflected in the composition of their seeds. BR action allowed seeds to become "equipped" with a higher amount of antioxidants and other important components. Increased accumulation of antioxidants may be important for at least two reasons. First, the accumulation of antioxidants may counteract seed aging (Pinzino et al., 1999). For example, carotenoids contribute to the antioxidant system in seeds, which functions to limit free radical-induced membrane deterioration and seed aging (Pinzino et al., 1999; Calucci et al., 2004). Second, antioxidants can act as a protective factor, which may be useful in the event of unfavorable environmental conditions during germination and early growth of next generation plants. Previously it was demonstrated that plants obtained from seeds borne on parents exposed to drought stress are more resistant to water deficit (Kalandyk et al., 2013). It cannot be ruled out that BR treatment of parent plants can also prepare the next generation of plants to better survive stress conditions, at least in the early stages of growth when a seed is the main nutritional source. These possibilities regarding the action of BR on seed aging or the stress resistance of next generation plants would require further and more detailed studies. Similarly, future studies are needed to explain the role of endogenous BR in the process of the regulation of seed filling. We should remember that in this aspect, studies using external BR application might not reflect normal physiological events. Anyway some clues for further studies of the role of BR in seeds are given. In addition, seed metabolic changes obtained as a result of external BR application may encourage the use of this growth regulator in practice to manipulate the concentration of individual components in seeds. Particularly interesting is the effect of BR on the accumulation of antioxidants (vitamins). Ascorbic acid (as vitamin C), tocopherols and tocotrienols (as vitamin E) and β-carotene (as provitamin A) are known as substances that affect the nutritional value of plants. They are essential in the human diet because our organisms do not produce them. Vitamin deficiency is a cause of serious disorders such as blindness and scurvy. In addition, these compounds may also have a therapeutic (anti-cancer) or cosmetic effect (Aggarwal et al., 2010; Taniguchi et al., 2012). Due to the important role they play, it is desirable to increase the content of these compounds in fruits and seeds of edible plants. Thus, these compounds are an object of interest of metabolomic engineering (Kumar et al., 2005; Paine et al., 2005; Ajiawi and Shintani, 2004). Therefore, it is worth knowing that the nutritional value of seeds can also be improved by hormonal regulation using brassinosteroids. This knowledge can be applied in practice in spraying or watering the plantations with BRs and modifying the chemical composition of selected species of leguminous seeds. It is worth mentioning that BRs are natural and environmentally friendly regulators, practically not harmful to mammals (Ramraj et al., 1997). On the other hand, the practical use of brassinosteroids would generate additional costs of the purchase of these compounds in cultivation. Currently, BRs commercially available in America or Europe are too expensive to use on a large scale. However, there are increasingly available cheaper BRs of Chinese production. Some countries (Cuba) have solved the problem by introducing BR analogues to agriculture and horticulture.

Materials and Methods

Plant material

The experiment was performed on two cultivars of pea (Roch and Wiato) and two cultivars of yellow lupine (Mister and Talar) derived from Poznan Plant Breeding Ltd. (Poland).

Plant growth

Plant culture in pots

Germinating seeds were put into Mitcherlich pots (diameter: 22 cm, height: 20 cm) filled with a mixture of soil:peat:sand in the ratio of 2:2:1 (5 seeds per pod). The seeds were sown in early April and watered once a week with Hoagland nutrient solution. Plants were cultured in an open vegetation hall with a transparent (plastic) roof during the vegetation season in 2010, in natural light conditions; latitude: 50°03' N, longitude: 19°55' E. Plants in the seedling stage were drenched with a preparation containing *Rhisobium* bacteria (Nitragina, Biofood, Poland). The plants were then cultured to the moment of flowering when 24-epibrassinolide was scheduled to be applied. In pea, it was the moment when 100% of plants had opened at least one flower. For lupine, it was the moment when 100% of plants had opened flowers on 2-4 of the lowest rows of bloom. Solutions of 24-epibrassinolide were then prepared. 24-epibrassinolide was purchased from Sigma-Aldrich (Poznan, Poland). The stock solution contained 4.1 mM BR_{27} in 50% ethanol. Working solutions used for the experiments (0.25 and 0.5 mg dm^{-3}) were prepared by diluting stock solution with distilled water. The range of concentrations of 24-epibrassinolide was chosen based on preliminary experiments (seedling growth response) as well as literature data (Ramraj et al., 1997; Janeczko et al., 2009; 2010). The controls were prepared as water solutions containing ethanol in the same amount as in the BR_{27} solution for each species and method of treatment. When the solutions were ready, the pots of flowering plants were randomly divided into four groups. For pea, it was the following groups: (1) plants for watering with solution BR_{27} (0.25 mg dm^{-3}); (2) plants for watering with water solution containing traces of ethanol (control); (3) plants for spraying with solution BR_{27} (0.5 mg dm^{-3}); (4) plants for spraying with water solution containing traces of ethanol (control). For lupine, it was these similar four groups, with the only difference being the concentration of BR_{27} used for plant spraying, which was 0.25 mg dm^{-3} for lupine. For both lupine and pea plants the volume of the foliar spraying was approximately 15 cm^3 per plant. The plants were sprayed in a manner so as to wet all the foliage and generative organs. All lupine and pea plants were watered with 25 cm^3 of BR_{27} solution per treated plant (application to the root system). Per one treatment there was four pods containing 5 plants each (together 20 plants per treatment). After treatment, the plants were maintained in the vegetation hall to obtain the yield and collect the seeds. After the harvest, the chemical composition of the yield was determined in order to study the influence of the treatments at flowering stage on the nutritional value of the obtained seeds (content of vitamins, proteins, fats and sugars).

Plant culture in the field

The field experiment was performed in the 2011 growing season (latitude: 50°03' N, and longitude: 19°55' E) on experimental plots at the Agricultural University of Krakow. The field was made up of brown soil of the class II wheat complex. The soil was acidic, low in nitrogen, high in assimilable phosphorus and potassium. Moreover, natural levels of copper, chromium, lead, nickel and zinc, and slightly elevated cadmium content were present. Weather conditions during the 2011 growing season: sum of rainfall [mm] per month: III – 15.2, IV – 77.7, V – 48.0, VI – 33.0, VII – 186.4, VIII – 73.1; average temperature per month [^oC]: III – 3.7, IV – 10.3, V – 13.6, VI – 18.2, VII – 17.6, VIII – 19.1. The experiment was carried out in a randomized block design where plants were growing in rows, directly in the soil. Germinating seeds of lupine and pea were sown according to the recommended date in early April. The rows were placed in two parallel belts (one belt was 2 m wide), separated by a 1 m path. Within the first belt, six blocks were prepared for lupine, the next six blocks for pea (there was a 1 m path between the species). Within the second belt, the order was opposite – first pea then lupine. Three blocks dedicated for each species consisted of one cultivar and the next three of the second cultivar. A single block had the dimensions: 3.6 m x 2 m. One block had 18 rows (15 plants in each row). The space between rows was 20 cm. The block contained: four rows dedicated to brassinosteroid spraying, four rows for sprayed control, one row of untreated plants, four rows for brassinosteroid watering, four rows for watered control and the last row with untreated plants. In the field experiment, the same BR concentrations and methods for plant treatments (spraying and watering) were used as described in the above pot experiment. Brassinosteroid application was done at the stage of plant flowering as described exactly in the "Plant culture in pots" section. During the vegetation season, plants were weeded several times and were fed once at the seedling stage with Azofoska fertilizer (40 g per m^2) (Inco Veritas SA, Poland). The composition of Azofoska fertilizer: NPK (MgO + SO3) 13.6%: 6.4%: 19.17% (4.5% + 23.0%) and Cu, Zn, Mn, Mo, Fe, B. Moreover plants at the seedling stage were drenched with a preparation containing *Rhisobium* bacteria (Nitragina, Biofood, Poland). After the harvest, the chemical composition of the yield was estimated by measurement of the content of vitamins, proteins, fats, and sugars in the collected seeds.

Chemical analysis

Grain samples were milled in a Unidrive1000 laboratory grinder (CAT, Germany). The fine powder was used for further analyses. All standards for analysis were bought from Sigma-Aldrich (Poznan, Poland).

Determination of tocopherols, tocotrienols, and β-carotene

The extraction of tocopherols, tocotrienols (tocochromanols), and β-carotene was done according to the method described by Janeczko et al. (2009). The obtained extract was then used for HPLC analyses.

Tocopherol and tocotrienol analysis was performed on an Agilent 1200 system with binary pump, autosampler, and fluorescence detector. The sample (0.02 cm^3) was injected into Zorbax Eclipse XDB 5 μm, 4.6 mm x 150 mm column (Agilent, USA), thermostated at 25°C. The linear gradient of A) methanol:acetonitrile:water (2.5:2.5:0.33 v/v) and B) acetonitrile:dichloromethan (1:1 v/v) was used (from 95% A to 30% A in 15 min) at a flow rate of 1 cm^3 per min. Tocochromanols were detected at the excitation wavelength

of 295 nm and the emission wavelength of 330 nm. The optimal parameters of fluorescent detection were chosen according to the absorption and emission spectra of standards (α-, γ-, δ-tocotrienol and α-, γ-, δ-tocopherol) acquired online. Determination of tocopherols was made for plant material from the pot and field experiment. Determination of tocotrienols was made for plant material from the field experiment.

β-Carotene was analyzed using an Agilent 1260 HPLC system with binary pump, thermostated autosampler at 10°C, and a diode array detector. The sample (0.02 cm^3) was injected on to analytical column Spherisorb ODS2, 3 μm, 4.6 mm x 150 mm (Waters, Ireland), thermostated at 25°C. The linear gradient of A) acetonitrile:water (2.5:0.33 v/v) and B) ethyl acetate was applied (from 0 min to 6.5 min 40% A, then to 8 min 20% A) at a flow rate of 1 cm^3 per min. A detector was set up at 450 nm. β-Carotene was used as a standard.

Ascorbic acid determination

Ascorbic acid content was determined by a modified CUPRAC method (Ozyürek et al., 2007). Pulverized samples (10 mg each) were extracted in 1 cm^3 of 3% sulfosalicylic acid for 30 min on a rotatory shaker (RL-2002, JWE, Poland). After centrifugation (2100 x *g* for 15 min at 10°C), 0.05 cm^3 of supernatant was transferred to a 96-well plate, containing 0.05 cm^3 0.01 M Cu(II) aqueous solution of $CuCl_2$ H_2O, 0.05 cm^3 7.5 mM neocuproine methanolic solution, and 0.05 cm^3 1 M amonium acetate buffer (pH 7.0). After five minutes absorbance at 450 nm it was measured on a Synergy II microplate reader (BioTek, USA). The amount of ascorbic acid was calculated with the use of a calibration curve made from ascorbic acid standard solutions.

Soluble (reducing and non-reducing) sugars determination

Sugars were analyzed spectrophotometrically according to Dubois et al. (1951) with modifications. Samples (5 mg each) were agitated in 1 cm^3 of deionized water for 15 min on a rotatory shaker (RL-2002, JWE, Poland). Then, samples were centrifuged at 2100 x *g* for 15 min and 0.04 cm^3 of supernatant was transferred to 7 cm^3 tubes containing 0.4 cm^3 of deionized water. Next, 0.4 cm^3 of 5% phenol and 2 cm^3 of concentrated sulfuric acid were added. The samples were incubated for 20 min and subsequently transferred to 96-well plates. The absorbance was read at 490 nm on a Synergy II microplate reader. The calibration was performed using glucose solutions.

Soluble proteins determination

Soluble proteins were estimated according to the Bradford (1976) method on 96-well plates. The absorbance was read at 595 nm on a Synergy II microplate reader.

Total fat determination

The total fat content was estimated in 0.2 g samples according to the method described by Bligh and Dyer (1959). Shortly, after extraction of the material, the chloroform phase was collected, evaporated and the total fat was estimated by weighing (gravimetrically).

Statistical analysis

Statistical significance was estimated based on a Student's test ($P \leq 0.05$). Objects were compared in pairs: BR_{27} spraying with proper control and BR_{27} watering with proper control separately for each chemical compound, species and cultivars. Chemical analysis was conducted in 3 replications (with 3 chemical repeats each). One replication was the sample taken from the ground seeds collected from 10 randomly selected plants. Statistical analysis of the results was carried out using the Statistica 10 (StatSoft, USA) program.

Conclusions

Two important findings are presented in the work. First, we show the possibility of using BR to manipulate plant seed content, which may be important from a nutritional point of view. BR_{27} modified the chemical composition of pea and lupine seeds, and particularly noteworthy was an increase in antioxidants (vitamins) level. Simultaneously, we show some mechanisms of BR action on the metabolism of seeds. The composition of seeds reflects in some way the metabolic response of the plant to brassinosteroid. Thus, the effects of the protective activity of BR in the form of the increased accumulation of nonenzymatic antioxidants are passed on to the next generation. BR action allows seeds to become "equipped" with antioxidants as a protective factor, which may be useful in the event of unfavorable environmental conditions during germination and early growth of next generation plants. On the other hand, seeds enriched in antioxidants can be more resistant to the aging processes. These hypotheses are interesting for further and more detailed studies.

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