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ORIGINAL ARTICLE

An estimation of the effects of synthetic auxin and cytokinin and the time of their application on some morphological and physiological characteristics of *Medicago* x *varia* T. Martyn

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Abstract The aim of the experiment was to determine the effects of synthetic auxin and cytokinin and the time of their application on some morphological and physiological characteristics of *Medicago* x *varia* T. Martyn grown under controlled conditions. The experiment was to check whether an application of exogenous hormones during vegetative and generative stages of the plant had an effect on above-ground mass development, on nitrate reductase activity and on plastid pigments content. Experiment factor was synthetic auxin and cytokinin and the date of their application. Auxin was applied in the form of a synthetic indole-3-butyric acid, while cytokinin was sprayed as synthetic 6-benzylaminopurine. The control plants were treated with distilled water. Depending on the experimental variant, spraying was applied at the sixth true leaf stage and at the first flower bud stage. The research showed that the response of the alfalfa plants to the application of cytokinin and auxin was not uniform. It seems that the most effective was the application of a mixture of them both but only during the vegetative stage.

Additionally, cytokinin caused an increase in plastid pigments content in alfalfa leaves. On the other hand, a mixture of auxin and cytokinin triggered the highest nitrate reductase activity in alfalfa roots and raised the ratio of total chlorophyll content to carotenoids. Synthetic auxin caused the decrease of the levels of most parameters compared to the control.

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1. Introduction

Recently there has been a growth of interest in investigating the influence of compounds having hormone properties on plants. The most important exogenous synthetic auxins are indole-3-butyric acid (IAA), indole-butric acid (IBA), beta naphtoxy acetic acid (NOA), 1-naphthaleneacetic acid (NAA), (2,4-dichlorophenoxy)propionic acid (2,4-D). Exogenous synthetic cytokinin forms are 6-benzylaminopurine (BAP) and 6-furfurylaminopurine or kinetin (Scacchi et al., 2009). Hormones take part in plant development in all stages from seed germination, through vegetative growth, flower induction to maturity and decomposition. Auxin and cytokinin are some of those important hormones having a cardinal role within plants (Karcz et al., 1996; McDonald, 1997; Reinecke et al., 1999; Copes and Mandel, 2000; Leyser, 2001; Nogalska and Czapla, 2002; Baluška et al., 2003; Benkova et al., 2003; Skutnik et al., 2004; Wodzicki, 2004; Costa et al., 2005; Heisler and Jönsson, 2006; Kramer and Bennett, 2006; Zhao, 2008; Willige et al., 2011). Some of the functions of auxin are cell elongation, fruit growth stimulation and development but also apical dominance regulation (McDonald, 1997). The basic functions of cytokinin are stimulation of cell division in meristems, prevention of senescence and elimination of apical dominance (Sujatha and Reddy, 1998). A characteristic feature differentiating both hormones is that they regulate photosynthesis and physiology in deferent parts of a plant (Mikos-Bielak, 2005).

A characteristic feature of plant hormones is their cooperation with other hormones. One of the examples can be exogenous auxin and cytokinin, in particular in larger concentrations, both stimulating the biosynthesis of ethylene in tissues (Lorteau et al., 2001; Khan et al., 2002). It proves that a lot of processes which are attributed to the effect of those hormones can be in fact the reaction of the plant to a high concentration of ethylene. Another example can be a mutual influence of cytokinin and auxin, for example, in the stimulation of cambium activity and the vascular tissue formation. It results in a better supply of tissues in photosynthesis products and raises immunity to stress factors, like water stress (Aldesuquy, 2000).

According to many publications (Galoch et al., 1996; Nahar and Ikeda, 2002), plant hormones, to a large extent, affect flower differentiation. Noden et al. (1990) say that there is a relation between the level of endogenous cytokinins and the number of flowers and pods shed by the plants of the *Fabaceae* family. He continues saying that spraying those plants with synthetic cytokinin prevents these processes by balancing hormonal activity. It turns out that the level of endogenous auxin in budding flowers is too low, which may be the cause of shedding flowers but also the cause of shedding of developing fruits. Spraying plants with auxin can change these processes. It is worth noting that there are plant enzymes which dissolve natural auxin (Rylott and Smith, 1990). Resse et al. (1995) and Rodrigo et al. (1997) attribute to hormones an important role in the transport and distribution of nutrients. Hormones affect the rise in acceptors and the rise in activities of enzymes loading and unloading phloem. Hormones play the role of signal substances to convey information about need of an acceptor.

The aim of the experiment was to determine the effects of synthetic auxin and cytokinin and the time of their application on some morphological and physiological characteristics of *Medicago* x *varia* T. Martyn grown under controlled conditions. The experiment was to check whether an application of exogenous hormones during vegetative and generative stages of the plant had an effect on above-ground mass development, on nitrate reductase activity and on plastid pigments content (chlorophyll a and b as well as carotenoids).

2. Materials and methods

2.1. The conditions of experiment establish

In March 2014, a pot experiment was conducted to grow *Medicago* x *varia* T. Martyn 'cv. Kometa' in a growing room at the Faculty of Natural Sciences of the Siedlce University of Natural Sciences and Humanities – Poland. The experiment conditions were: temperature 24 ± 2/16 ± 2 °C; photoperiod 16/8 h; light intensity of 200 μmol m^{-2} s^{-1} achieved through the use of high-pressure sodium lamps; and humidity 40%. The experiment was completely randomised, with four replications with the control subject. The entire experiment was conducted in 40 pots, four pots for each variant and 3 plants in each pot. The experimental factor consisted of two growth regulators – synthetic auxin and synthetic cytokinin.

The pots were filled with 5 kg of soil each. The soil used in the experiment was composed of loamy medium sand, III soil valuation class, taken from the arable topsoil level. It was characterised with a very high content of assimilable phosphorus and magnesium, a high content of potassium, copper and zinc, and a medium content of boron, manganese and iron (Table 1).

Alfalfa seeds were sown in mid-March to a depth of 2–3 cm. After the seeds germinated, 3 representative plants in each pot were left for further research. During their growth, the alfalfa plants were sprayed with the growth regulators according to the methodology data presented in Table 2. Auxin was applied in the form of a synthetic indole-3-butyric acid (IBA – concentration of 30 mg dm^{-3}), while cytokinin was sprayed as synthetic 6-benzylaminopurine (BAP – concentration of 30 mg dm^{-3}). The control plants were treated with distilled water. The plants were sprayed until dip-off with 0.20 dm^3 of spray liquid per pot. Depending on the experimental variant, spraying was applied at the sixth true leaf stage (F_1) and at the first flower bud stage (F_2).

The experiment was replicated four times. When the flowers faded plants were harvested and the biomass was fractionated into that of roots, stalks, leaves and inflorescence.

2.2. Analysis of morphological and physiological traits

The following features were measured in the experiment: the number of stomata (pieces mm^{-2}), the length of stomata (μm), the breadth of stomata (μm), the length of stomatal pores (μm), the stalk length [cm], the number of shoots per plant (pieces), the leaf number per shoot (pieces) and the diameter of root collars (mm). Moreover, the total mass of one plant was determined (g DM), the mass of a shoot (g DM), the mass of leaves per shoot (g DM), the mass of inflorescence per shoot (g DM) and the mass of the root system (g DM).

Measurements of stomata were taken after the harvest, choosing randomly ten leaves from each plant. It was done

Table 1 Chemical composition of soil.

pH in KCL	Humus [%]	C_{org} [g kg^{-1}]	Dry matter [%]	Humidity [%]
6.3	3.0	17.1	86	12
Content of mineral N [mg kg^{-1} DM]		Total content of macroelements [g kg^{-1} DM]		
$N\text{-}NO_3$	$N\text{-}NH_4$	P	K	Mg
1.4	60.9	0.48	0.13	0.10
Total content of microelements [mg kg^{-1} DM]				
B	Mn	Cu	Zn	Fe
2.3	191	8.7	22.3	1570

Table 2 Methodological data.

Treatment	Type of spray	Phase	
		F1 Sixth true leaf stage	F2 First flower bud stage
K	Distilled water	Water – 0.2 dm^3 pot^{-1}	Water – 0.2 dm^3 pot^{-1}
AF_1	Auxin	IBA – 0.2 dm^3 pot^{-1}	Water – 0.2 dm^3 pot^{-1}
CF_1	Cytokinin	BAP – 0.20 dm^3 pot^{-1}	Water – 0.2 dm^3 pot^{-1}
ACF_1	Auxin + cytokinin	IBA + BAP – 0.2 dm^3 pot^{-1}	Water – 0.2 dm^3 pot^{-1}
AF_2	Auxin	Water – 0.2 dm^3 pot^{-1}	IBA – 0.2 dm^3 pot^{-1}
CF_2	Cytokinin	Water – 0.2 dm^3 pot^{-1}	BAP – 0.2 dm^3 pot^{-1}
ACF_2	Auxin + cytokinin	Water – 0.2 dm^3 pot^{-1}	IBA + BAP – 0.2 dm^3 pot^{-1}

K – control, A – auxin (indole-3-butyric acid), C – cytokinin (6-benzylaminopurine) AC – auxin + cytokinin.

with the microscope Olipmus CX 41 and with the DP – soft program for image processing and analysis. The raw material for the research was the upper epidermis taken from the middle part of the leaf blade (Braune et al., 1975). All measurements were taken with the images magnified 400 times.

After withering of the plants, the collection and fractionation of biomass for roots, leaves, stems and inflorescences was conducted. In stems, leaves and roots nitrate reductase activity was determined with the *in vitro* method according to Jaworski (1971). Moreover, plastid pigment content was marked in leaves. Chlorophyll a and b were marked with the method by Arnon et al. (1956) as modified by Lichtenthaler and Wellburn (1983), and the content of carotenoids – using the method by Hager and Mayer-Berthenrath (1966). Plant material for the marking was collected from each plant at the full flowering stage (50% of open flowers). As for the pigments, the optical density of the obtained supernatants was determined with the Marcel Mini spectrophotometer with wavelengths: 440, 465 and 663 nm. Next, the results were calculated according to the following formulas:

– chlorophyll a content: $[12.7(E663) - 2.69(E645)] \times w/v$;
– chlorophyll b content: $[22.9(E645) - 4.68(E663)] \times w/v$;
– chlorophyll a + b content: $[20.2(E645) - 8.02(E663)] \times w/v$;
– carotenoid content: $[4.16(E440) - 0.89(E663)] \times w/v$;

where: E – extinction at a particular wavelength; v – amount of 80% acetone [cm^3] used for extraction; w – sample weight [g].

Tukey's test was used to find means that were significantly different from each other, at the significance level of $NIR_{0.05}$.

3. Results

3.1. Stomatal parameters and plant biometrics

The results of the research (Table 3) shows that application of synthetic hormones to the alfalfa plants did not affect the number and length of stomata in the leaf epidermis. However, it was noted that application of synthetic auxin and cytokinin had an impact on the breadth of stomata, increasing it by 33% on average. On the other hand, statistically significant growth in the length of stomata pores was noted when synthetic cytokinin was applied (by 20.6% on average) and the mixture of auxin and cytokinin caused the same result (a growth by 13.7% on average). Analysis of growth and development stages of the plants clearly indicated that application of hormones during bud formation did not affect development of stomata in the leaf epidermis of the alfalfa plant. The data presented in Tables 4 and 5 show that after the application of synthetic auxin and cytokinin and the mixture of the two hormones the growth and development of particular parts of the alfalfa plants varied considerably. Auxin, when applied during the stage of sixth true leaves, caused the highest growth in the length of stalks, in the diameter of the root collar and the highest growth of dry matter. Cytokinin applied during the vegetative stage increased the number of leaves but made them grow smaller, which resulted in a smaller mass compared to other experiment objects. Because cytokinin caused shortening of the stalks, the mass of stalks was smaller too. In turn, the mixture of auxin and cytokinin diminished the mass of inflorescence. The same mixture when applied during the vegetative stage caused an increase in the number of shoots (on average

Table 3 Stomatal parameters of *Medicago* x *varia* T. Martyn in relation to the time of application and kind of growth hormones applied.

Kind of hormones	Date of spray		Mean
	F1	F2	
Number of stomata (pieces mm^{-2})			
K	71^{Aa}	73^{Aa}	72^{A}
A	80^{Aa}	78^{Aa}	79^{A}
C	81^{Aa}	84^{Aa}	83^{A}
AC	74^{Aa}	82^{Aa}	78^{A}
Mean	77^{a}	79^{a}	
Stomatal length (µm)			
K	24.1^{Aa}	22.8^{Aa}	23.5^{A}
A	23.2^{Aa}	24.4^{Aa}	23.8^{A}
C	24.6^{Aa}	21.9^{Aa}	23.3^{A}
AC	25.0^{Aa}	21.1^{Aa}	23.1^{A}
Mean	24.2^{a}	22.6^{a}	
Stomatal breadth (µm)			
K	11.8^{Ba}	12.0^{Aa}	11.9^{B}
A	14.9^{Aa}	11.6^{Ab}	13.3^{A}
C	16.2^{Aa}	11.1^{Ab}	13.7^{A}
AC	16.1^{Aa}	11.7^{Ab}	13.9^{A}
Mean	14.8^{a}	11.6^{b}	
Length of the stomatal pore (µm)			
K	13.1^{Ba}	12.9^{Aa}	13.0^{B}
A	13.4^{Ba}	13.1^{Aa}	13.3^{B}
C	15.8^{Aa}	13.3^{Ab}	14.6^{A}
AC	14.9^{Aa}	13.2^{Ab}	14.1^{A}
Mean	14.3^{a}	13.1^{b}	

– Means in lines marked with the same small letters do not differ significantly
– Means in columns marked with the same capital letters do not differ significantly

K – control, A – auxin (indole-3-butyric acid), C – cytokinin (6-benzylaminopurine), AC – auxin + cytokinin, F1 – sixth true leaf stage, F2 – first flower bud stage.

Table 4 Length of stocks of *Medicago* x *varia* T. Martyn, the number of shoots and leaves and the diameter of the root collar in relation to the kind of hormone applied and time of application.

Kind of hormones	Date of spray		Mean
	F1	F2	
Length of stalks (cm)			
K	46.9^{Ba}	42.3^{Aa}	44.6^{B}
A	57.1^{Aa}	41.2^{Aa}	49.2^{AB}
C	37.9^{Cb}	47.9^{Aa}	42.9^{B}
AC	56.6^{Aa}	46.8^{Ab}	51.7^{A}
Mean	49.6^{a}	44.6^{b}	
Number of shoots per plant (pieces)			
K	12.0^{Ba}	11.0^{Aa}	11.5^{B}
A	12.0^{Ba}	10.0^{Ab}	11.0^{B}
C	8.0^{Cb}	11.0^{Aa}	9.5^{B}
AC	18.0^{Aa}	10.0^{Ab}	14.0^{A}
Mean	12.5^{a}	10.5^{b}	
Number of leaves per shoot (pieces)			
K	37.3^{Ba}	36.7^{Aa}	37.0^{A}
A	23.5^{Bb}	49.3^{Aa}	36.4^{A}
C	43.2^{Aa}	24.0^{Bb}	33.6^{A}
AC	25.3^{Bb}	45.1^{Aa}	35.2^{A}
Mean	32.3^{a}	38.8^{a}	
Diameter of root collar (mm)			
K	3.5^{Ca}	3.9^{Aa}	3.7^{B}
A	6.4^{Aa}	3.8^{Ab}	5.1^{A}
C	3.6^{Ca}	3.8^{Aa}	3.7^{B}
AC	4.9^{Ba}	3.7^{Ab}	4.3^{AB}
Mean	4.6^{a}	3.8^{b}	

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– Means in columns marked with the same capital letters do not differ significantly

K – control, A – auxin (indole-3-butyric acid), C – cytokinin (6-benzylaminopurine), AC – auxin + cytokinin, F1 – sixth true leaf stage, F2 – first flower bud stage.

21.7% compared to the control plot) and an increase in the mass of well developed shoots (on average 47.8% compared to the control plot).

3.2. Nitrate reductase activity in different parts of plants

The research (Table 6) has shown that nitrate reductase activity in the control subject plants was 32.1 µmol NO_2^- g^{-1} fresh weight in leaves, 8.40 µmol NO_2^- g^{-1} fresh weight in stems, and 4.20 µmol NO_2^- g^{-1} fresh weight in roots. Nitrate reductase activity in leaves and stems increased or decreased, depending on the combination of hormones and the stage at which spraying was applied. A similar effect of auxin on nitrate reductase activity was also observed in the fresh weight of alfalfa stems. On the other hand, irrespective of the hormone type and the spraying number and stage, nitrate reductase activity in roots was higher compared to the control. The highest activity (66.7%) was observed for the plants sprayed only once with a mixture of auxin and cytokinin at the first flower bud stage – F_2.

3.3. Plastid pigments content in leaf blades

The study (Table 7) showed that spraying the plants with hormones triggered an increase in the chlorophyll a content in alfalfa leaf blades. Additionally, application of cytokinin only at the sixth true leaf stage (F_1) or only at the first flower bud stage (F_2) resulted in a substantial increase in the content of chlorophyll a, about 147% and 132% respectively, in comparison to the control. The weakest effect (from 6.64% to 23.9% relative to the control) was observed after auxin spraying. It should be noted that it led to a decrease in the content of chlorophyll b. The sharpest drop (48.3%) compared to the control was observed in the plants sprayed once with auxin at the sixth true leaf stage – F_1. As for the total chlorophyll (368–384 mg 100 g^{-1} fresh weight), the best results were obtained after applying cytokinin at both stages of alfalfa development. Similarly, cytokinin spraying resulted in the largest increase in carotenoid content (Table 8). Spraying the plants with this hormone at the sixth true leaf stage (F_1) and at the first flower bud stage (F_2) caused a 107% rise in carote-

Table 5 Mass of different parts of *Medicago* x *varia* T. Martyn in relation to the kind of the hormone applied and the time of application.

Kind of hormones	Date of spray		Mean
	F1	F2	
Total dry matter (g DM)			
K	33.3^{ABa}	30.9^{ABa}	32.1^{A}
A	29.2^{Bb}	44.7^{Aa}	37.0^{A}
C	20.8^{Ba}	24.7^{Ba}	22.8^{B}
AC	39.7^{Aa}	28.8^{Bb}	34.3^{A}
Mean	30.8^{a}	32.3^{a}	
Mass of a shoot per plant (g DM)			
K	11.5^{Ba}	10.5^{BCa}	11.0^{B}
A	11.7^{Bb}	20.0^{Aa}	15.9^{A}
C	8.5^{Cb}	12.0^{Ba}	10.3^{B}
AC	17.0^{Aa}	9.61^{Cb}	13.3^{AB}
Mean	12.2^{a}	13.0^{a}	
Mass of leaves per shoot (g DM)			
K	16.8^{Aa}	15.1^{Ba}	16.0^{A}
A	11.3^{Bb}	19.9^{Aa}	15.6^{A}
C	5.70^{Cb}	8.30^{Ca}	7.00^{B}
AC	18.4^{Aa}	12.4^{Bb}	15.4^{A}
Mean	13.0^{a}	13.9^{a}	
Mass of inflorescence per shoot (g DM)			
K	1.50^{Ba}	1.40^{Aa}	1.45^{B}
A	0.80^{Ca}	0.20^{Bb}	0.50^{C}
C	2.70^{Aa}	1.30^{Ab}	2.00^{A}
AC	0.20^{Da}	0.20^{Ba}	0.20^{C}
Mean	1.30^{a}	0.80^{b}	
Mass of the root system (g DM)			
K	3.5^{Ba}	3.9^{Aa}	3.70^{B}
A	5.4^{Ab}	4.6^{Aa}	5.00^{A}
C	3.9^{Bb}	3.1^{Ba}	3.50^{B}
AC	4.1^{ABa}	4.6^{Aa}	4.35^{AB}
Mean	4.23^{a}	4.10^{a}	

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K – control, A – auxin (indole-3-butyric acid), C – cytokinin (6-benzylaminopurine), AC – auxin + cytokinin, F1 – sixth true leaf stage, F2 – first flower bud stage.

noid content compared to the control. Application of cytokinin at the F_1 stage contributed to a 95.3% increase, whereas when applied at the F_2 stage, it led to a 74.5% change in the value of this particular characteristic. Auxin spraying had an influence on the decline in carotenoids, but only in the variant with a single spraying at the F_1 stage. The use of auxin only at the first flower bud stage improved pigment content in the plant material by 5.11%. The most dramatic drops (6.80%) in carotenoids were observed for a mixture of auxin and cytokinin used only at the sixth true leaf stage (F_1). The highest ratio of total chlorophyll to carotenoids (13.3 on average) was found for the plant material treated with a mixture of auxin and cytokinin, regardless of the number of sprayings and their stage. In contrast, values lower than the control were achieved with auxin.

Table 6 Nitrate reductase activity in leaves, stems and roots of *Medicago* x *varia* T. Martyn according to the development phase of the plant and the type of spray.

Kind of hormones	Date of spray		Mean
	F1	F2	
Leaves [µmol NO_2^- g^{-1} fresh weigh]			
K	32.1^{Ba}	30.9^{Ba}	31.5^{B}
A	31.7^{Ba}	30.2^{Ba}	31.5^{B}
C	43.0^{Aa}	45.0^{Aa}	44.0^{A}
AC	50.7^{Aa}	46.1^{Aa}	48.4^{A}
Mean	39.2^{a}	38.1^{a}	
Stems [µmol NO_2^- g^{-1} fresh weigh]			
K	8.40^{Ca}	8.64^{Ba}	8.52^{C}
A	8.30^{Ca}	8.10^{Ba}	8.21^{C}
C	10.7^{Bb}	12.3^{Aa}	11.5^{B}
AC	13.4^{Aa}	13.9^{Aa}	13.7^{A}
Mean	10.2^{a}	10.7^{a}	
Roots [µmol NO_2^- g^{-1} fresh weigh]			
K	4.20^{Ba}	4.25^{Ba}	4.23^{C}
A	6.80^{Aa}	6.30^{Aa}	6.55^{A}
C	5.20^{Ba}	5.70^{Ba}	5.45^{B}
AC	6.40^{Ba}	7.00^{Ab}	6.70^{A}
Mean	6.10^{a}	5.81^{a}	

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– Means in columns marked with the same capital letters do not differ significantly

K – control, A – auxin (indole-3-butyric acid), C – cytokinin (6-benzylaminopurine), AC – auxin + cytokinin, F1 – sixth true leaf stage, F2 – first flower bud stage.

4. Discussion

Controlling gas exchange and, at the same time, photosynthesis, stomata have an impact on plant growth and development (Jones, 1998). Klamkowski et al. (2008) say that a higher leaf stomatal density is related to a more dynamic gas exchange. Thus, the number of stomata is related to stomatal conductance, which means that a higher number results in a higher rate of photosynthesis and transpiration (Klamkowski et al., 2008). In our study application of synthetic hormones to the alfalfa plants did not affect the number and length of stomata in the leaf epidermis. Also analysis of growth and development stages of the plants clearly indicated that application of hormones during bud formation did not affect development of stomata in the leaf epidermis of the alfalfa plant. Hormones regulate plant growth and development and, at the same time, the intake of nutrients. Thus, hormones are endogenous substances regulating distribution and accumulation of nutrients (Panwar et al., 1990; Nowak and Ciećko, 1991; Nowak et al., 1997; Czapla et al., 2003). Nowak and Ciećko (1991) suggest that a higher biomass growth and a higher concentration of some minerals in the above-ground parts of plants after application of synthetic hormones are caused by a bigger growth of the root system, in particular because of the lengthening of the root hair zone (Svenson, 1991; Meuwly and Pilet, 1991; Ali et al., 2008). In consequence of this lengthening the intake of ground water and intake of nutrients are both higher. Reinhardt et al. (2000)

Table 7 Content of a and b chlorophyll in *Medicago* x *varia* T. Martyn leaf depending on the development stage of plants and the type of spray.

Kind of hormones	Date of spray		Mean
	F1	F2	
Chlorophyll a [mg 100 g^{-1} fresh wright]			
K	108^{Ca}	111^{Ca}	109^{C}
A	115^{Cb}	134^{Ca}	125^{C}
C	268^{Aa}	251^{Aa}	260^{A}
AC	202^{Ba}	213^{Bb}	208^{B}
Mean	173^{a}	177^{a}	
Chlorophyll b [mg 100 g^{-1} fresh wright]			
K	80.4^{Ba}	82.3^{Ca}	81.4^{B}
A	41.6^{Cb}	43.0^{Ca}	42.3^{C}
C	116^{Ab}	117^{Aa}	116^{A}
AC	85.9^{Ba}	78.7^{Bb}	82.3^{B}
Mean	81.7^{a}	80.3^{a}	
Chlorophyll a + b [mg 100 g^{-1} fresh wright]			
K	188^{Ca}	193^{Ca}	190^{C}
A	157^{Cb}	177^{Ca}	167^{C}
C	384^{Ab}	368^{Aa}	376^{A}
AC	288^{Ba}	292^{Bb}	290^{B}
Mean	254^{a}	257^{a}	

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K – control, A – auxin (indole-3-butyric acid), C – cytokinin (6-benzylaminopurine), AC – auxin + cytokinin, F1 – sixth true leaf stage, F2 – first flower bud stage.

Table 8 The content of carotenoids in the leaves of *Medicago* x *varia* T. Martyn, and the ratio of the content of total chlorophyll to carotenoids depending to the development phase of the plant and the type of spray.

Kind of hormones	Date of spray		Mean
	F1	F2	
Carotenoids [mg 100 g^{-1} fresh wright]			
K	23.5^{Ba}	25.0^{Ba}	24.3^{B}
A	22.6^{Bb}	24.7^{Ba}	23.7^{B}
C	45.9^{Aa}	41.0^{Aa}	43.5^{A}
AC	21.9^{Ba}	22.3^{Ba}	22.1^{B}
Mean	28.5^{a}	28.3^{a}	
Chlorophyll a + b/carotenoids			
K	8.00^{BCa}	7.72^{Ba}	7.86^{B}
A	6.95^{Cb}	7.17^{Ba}	7.06^{B}
C	8.37^{Ba}	8.98^{Ba}	8.68^{B}
AC	13.2^{Aa}	13.4^{Aa}	13.3^{A}
Mean	9.13^{a}	9.32^{a}	

– Means in lines marked with the same small letters do not differ significantly
– Means in columns marked with the same capital letters do not differ significantly

K – control, A – auxin (indole-3-butyric acid), C – cytokinin (6-benzylaminopurine), AC – auxin + cytokinin, F1 – sixth true leaf stage, F2 – first flower bud stage.

and Friml (2003) say that auxin is the key factor here since it functions as a signal informing about physiological processes in cells and their growing demand for nutrients. Moreover, cytokinin and auxin stimulate the activity of cambium and forming of phloem, making it possible for different kinds of nutrients to be delivered to all parts of a plant (Reinhardt et al., 2000; Cho et al., 2002; Friml, 2003). In our study auxin, when applied during the stage of sixth true leaves, caused the highest growth in the length of stalks, in the diameter of the root collar and the highest growth of dry matter. Cytokinin applied during the vegetative stage increased the number of leaves but made them grow smaller. Rylott and Smith (1990), Weijers and Jürgens (2004) say that treating plants with synthetic auxin may contribute to the growing competition between vegetative and reproductive organs. Research done by Pandey et al. (2003) confirmed that. They used indolilo-acetic acid, IAA, on cotton plants and noted a considerable growth of inflorescence. In turn, Nagel et al. (2001) suggest that application of synthetic cytokinin resulted in better vascularity of tissues and, in consequence, a better transport of photosynthetic products form vegetative to reproductive organs. At the same time the concentration of photosynthetic products in vessels of plants tested was higher. After the research on the pigeon pea with BAP, 6-benzylaminopurine, applied, Barclay and McDavid (1998) noted that its pods grew much thicker and as a result their mass was higher. In our study nitrate reductase activity in leaves and stems increased or decreased, depending on the combination of hormones and the stage at which spraying was applied. A similar effect of auxin on nitrate reductase activity was also observed in the fresh weight of alfalfa stems. Irrespective of the hormone type and the spraying number and stage, nitrate reductase activity in roots was higher compared to the control.

The lowest nitrate reductase activity in the tissues of alfalfa roots compared to the leaf and stem tissues was also observed by other authors (Vasileva and Ilieva, 2007, 2011; Ilieva and Vasileva, 2013). It should be pointed out, however, that the obtained values of the activity of the examined enzyme were within the limits described by the available scientific literature. In our study nitrate reductase activity in leaves and stems increased or decreased, depending on the combination of hormones and the stage at which spraying was applied. A similar effect of auxin on nitrate reductase activity was also observed in the fresh weight of alfalfa stems. Irrespective of the hormone type and the spraying number and stage, nitrate reductase activity in roots was higher compared to the control. Fu et al. (2000) reported that when plants from the *Fabaceae* family age, chlorophyll and carotenoid content in tissues decreases. The use of exogenous cytokinin may increase the content of chlorophyll in senescent leaf tissues, because it slows down chlorophyll degradation and delays the aging process. In their studies on the influence of BAP on cabbage, Costa et al. (2005) found that it slows the degradation of the total chlorophyll compared to the control plants. Authors also focused on determining the activity of enzymes taking part in chlorophyll degradation, such as chlorophyllase and magnesium dechelatase. It turned out that there was a significant drop in the activity of these enzymes in the plants sprayed with BAP compared to the control plants. Using two types of synthetic auxins – IBA and NAA (1-naphthaleneacetic acid) – and mixtures thereof (all in a concentration of 20 mg dm^{-3}), Czapla et al. (2003) observed the largest effects when using IBA. On

the other hand, Nahar and Ikeda (2002) sprayed soybean plants with auxin – ethyl-5-chloro-3-indazolyl acid – and found an average of 23% increase in the analysed features compared to the control. The positive effect of plant hormones on plants was also reported by Barclay and McDavid (1998) and Czapla et al. (2003).

5. Conclusions

No matter what the time of synthetic hormone application was, the alfalfa responded with broader stomata whereas the length of the stomatal pore was the highest in objects with synthetic cytokinin applied and with a mixture of cytokinin and auxin applied. The highest biomass of the above-ground parts was noted for plants treated with the mixture of auxin and cytokinin. The plants also responded with the highest number and length of shoots. Statistical analysis showed that the time of spray affected the length and number of shoots, the diameter of the collar root and the mass of inflorescence. The value of those features was higher for plants sprayed at the stage of sixth true leaves (vegetative stage). It seems that the most effective was the application of a mixture of them both but only during the vegetative stage. Treating the plants with auxin alone decreased the activity of this enzyme in stems and roots compared to the control. Spraying the plants with exogenous auxin and cytokinin contributed to an increase in chlorophyll content in alfalfa leaves. The content of chlorophyll b and a + b in the plant material was very diverse. The highest concentration of carotenoids was found in the alfalfa leaves sprayed twice with cytokinin. Auxin decreased their content. Spraying alfalfa with a mixture of auxin and cytokinin had the most visible effects only on the ratio of total chlorophyll to carotenoids, as the ratio value increased by 65%.

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References

Aldesuquy, H.S., 2000. Effect of indol-3-yl acetic acid on photosynthetic characteristics of wheat flag leaf during grain filling. Photosynthetica 38 (1), 135–141.

Ali, B., Hayat, S., Hasan, S., Ahmad, A., 2008. A comparative effect of IAA and 4-Cl-IAA on growth, nodulation and nitrogen fixation in *Vigna radiata* (L.). Acta Pysiol. Plant. 30, 35–41.

Arnon, D.J., Allen, M.B., Whatley, F., 1956. Photosynthesis by isolated chloroplast. IV General concept and comparison of three photochemical reactions. Biochim. Biophys. Acta 20, 449–461.

Baluška, F., Šamaj, J., Menzel, D., 2003. Polar transport of auxin: carrier – mediated flux across the plasma membrane or neurotransmitter – like secretion? Trends Cell Biol. 13 (6), 282–285.

Barclay, G.F., McDavid, C.R., 1998. Effect of benzyloaminopurine on fruit set and seed development in pigeon pea (*Cajanus cajan*). Sci. Hort. 72, 81–86.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., Friml, J., 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115 (26), 591–602.

Braune, W., Leman, A., Taubert, H., 1975. Methods [in Praktikum the anatomy of plants]. PWN Warsaw, 298–317.

Cho, T., Suh, K.S., Park, H.K., Wodd, A., 2002. Impact of 2,4-DP and BAP upon pod set and seed yield in soybean treated at reproductive stages. Plant Growth Regul. 36, 215–221.

Copes, D.L., Mandel, N.L., 2000. Effects of IBA and NAA treatments on rooting Douglas-fir stem cuttings. New Forest. 20, 249–257.

Costa, M.L., Civello, P.M., Chaves, A.R., Martinez, G.A., 2005. Effect of ethephon and 6-benzylaminopuryne on chlorophyll degrading enzymes and peroxidase-linked chlorophyll bleaching during post-harvest senescence of broccoli (*Brassica oleracea* L.) at 20 °C. Postharvest Biol. Technol. 35, 191–199.

Czapla, J., Nogalska, A., Stasiulewicz, L., 2003. Synthetic auxin effect on the yield and the mineral soybeans. Acta Sci. Pol. Agric. 2 (1), 123–131 (In Polish).

Friml, J., 2003. Auxin transport – shaping the plant. Curr. Opin. Plant Biol. 6 (1), 7–12.

Fu, J., Huang, B., Zhang, G., 2000. Physiological and biochemical changes during seed filling in relation to leaf senescence in soybean. Biol. Plant. 43 (4), 545–548.

Galoch, E., Burkacka-Łakajtus, E., Kopcewicz, J., 1996. Effect of cytokinins on flower differentiation in cultured plants of *Pharbitis nil* Chois. Acta Pysiol. Plant. 18 (3), 223–227.

Hager, A., Mayer-Berthenrath, T., 1966. Die isolierung und quantative bestimung der carotenoide und chlorophyll von Blatern, algen und isolierten chloroplasten mit hilfe dunnschichtchromatographischer methoden. Planta Berlin 69, 198–217.

Heisler, M.G., Jönsson, H., 2006. Modeling auxin transport and plant development. J. Plant Growth Regul. 25, 302–312.

Ilieva, A., Vasileva, V., 2013. Effect of mineral fertilization and manure on some characteristics in Alfalfa. In: Medicago sativa, L. (Ed.), World Appl. Sci. J., vol. 26, pp. 630–635.

Jaworski, E., 1971. Nitrate reductase assay in intact plant tissues. Biochem. Biophys. Res. Commun. 13 (6), 1274–1279.

Jones, A.M., 1998. Auxin transport: down and out and up again. Science 282, 2201–2202.

Karcz, A., Nowak, G., Wierzbowska, J., Rotkiewicz, M., 1996. Effect of IAA and 4-CL-IAA on growth rate in maize coleoptile segments. Acta Physiol. Plant. 21 (2), 133–139.

Khan, N.A., Khan, M., Ansari, H.R., 2002. Auxin and defoliation effects on photosynthesis and ethylene evolution in mustard. Sci. Hort. 96, 43–51.

Klamkowski, K., Treder, W., Marasek, A., Borkowska, B., 2008. Characteristics of stomata and gas exchanges of strawberry plants, depending on growth conditions. Adv. Agric. Sci. Probl. 524, 499–509 (In Polish).

Kramer, M.E., Bennett, J., 2006. Auxin transport: a field in flux. Trends Plant Sci. 11 (8), 383–386.

Leyser, O., 2001. Auxin signalling: the beginning, the middle and the end. Curr. Opin. Biol. 4, 382–386.

Lichtenthaler, H.K., Wellburn, A.R., 1983. Determinations of total carotenoids and chlorophyll a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 11, 591–592.

Lorteau, M.A., Ferguson, B.J., Guinel, F.C., 2001. Effect of cytokinin on ethylene production and nodulation in pea (*Pisum sativum*) cv. Sparkle. Physiol. Plant. 112, 421–428.

McDonald, H., 1997. Auxin perception and signal transduction. Physiol. Plant. 100, 423–430.

Meuwly, P., Pilet, P., 1991. Local treatment with indole-3-acetic acid induces differential growth responses in *Zea mays* L. root. Planta 185, 58–64.

Mikos-Bielak, M., 2005. Exogenous growth regulators in potato. Ann. UMCS Sect. E 60, 281–292 (In Polish).

Nagel, L., Brewster, R., Riedell, W.E., Reese, R.N., 2001. Cytokinin regulation of flower and pod set soybeans (*Glycine max* L. Merr.). Ann. Bot. 88, 27–31.

Nahar, B.S., Ikeda, T., 2002. Effect of different concentrations of Figaron on production and abscission of reproductive organs,

growth, and yield in soybean (*Glycine max* L.). Field Crops Res. 78, 41–50.

Noden, L.D., Santokh, S., Letham, D.S., 1990. Correlation of xylem sap cytokinin levels with monocarpic senescence in soybean. Plant Physiol. 93, 33–39.

Nogalska, A., Czapla, J., 2002. Yielding of spring barley depending on the application of growth regulators, and mixtures thereof with magnesium sulfate. Pol. J. Nat. Sci. 12 (3), 37–51 (In Polish).

Nowak, G., Ciećko, Z., 1991. Effect of GA3 on the yield and value of forage turnip. Zesz. Nauk. AR Kraków 262, 325–331 (In Polish).

Nowak, G.A., Klasa, A., Wierzbowska, J., Rotkiewicz, M., 1997. Yield and macronutrient content in faba bean plants in the conditions of use retardant and plant hormones. Biul. IHAR 201, 289–294 (In Polish).

Pandey, D.M., Goswami, C.L., Kumar, B., 2003. Physiological effects of plant hormones in cotton under drought. Biol. Plant. 47 (4), 535–540.

Panwar, J.D.S., Abbas, S., Raum, S., 1990. Effects of benzol-amino purine and girdling site on photosynthesis translocation and nitrogen fixation in Y-shaped mungbean. Indian J. Plant Physiol. 33 (1), 16–20.

Reinecke, D.M., Ozga, J.A., Ilić, N., Magnus, V., Kojić-Prodić, B., 1999. Molecular properties of 4-substituted indole-3-acetic acids affecting pea pericarp elongation. Plant Growth Regul. 27, 39–48.

Reinhardt, D., Mandel, T., Kuhlemeier, C., 2000. Auxin regulates the initiation and radial position of plant lateral organs. Plant Cell 12, 507–518.

Resse, R.N., Dybing, C.D., White, C.A., Page, S.M., Larson, J.E., 1995. Expression of vegetative storage protein (VSP-β) in soybean raceme tissues in response to flower set. J. Exp. Bot. 46, 957–964.

Rodrigo, M.J., Garcia-Martinez, J., Santes, C.M., Gaskin, P., Hedden, P., 1997. The role of gibberellins A1 and A3 in fruit growth of *Pisum sativum* L. and the identification of gibberellins A4 and A7 in young seeds. Planta 201, 446–455.

Rylott, P.D., Smith, M.L., 1990. Effects of applied growth substances on pod set in broad beans (*Vicia faba var. major*). J. Agric. Sci. 114, 41–47.

Scacchi, E., Osmont, K.S., Beuchat, J., Salinas, P., Navarrete-Gómez, M., Trigueros, M., Ferrándiz, C., Hardtke, C.S., 2009. Dynamic auxin-responsive plasma membrane-to-nucleus movement of Arabidopsis BRX. Development 136 (12), 2059–2067. http://dx.doi.org/10.1242/dev.035444. ISSN: 0950-1991.

Skutnik, E., Rabiza-Świder, J., Wachowicz, M., Łukaszewska, A.J., 2004. Senescence of cut leaves of *Zantedeschia aethiopica* and *Z. elliottiana*. Part I. Chlorophyll degradation. Acta Sci. Pol. Hortorum Cultus 3 (2), 57–65.

Sujatha, M., Reddy, T.P., 1998. Differential cytokinin effect on stimulation of in vitro shoot proliferation from meristematic explants of castor (*Ricinus comminos* L.). Plant Cell Rep. 17, 561–566.

Svenson, S.E., 1991. Rooting and lateral shoot elongation of Verbena following benzylaminopurine application. Hort. Sci. 26, 391–392.

Vasileva, V., Ilieva, A., 2007. Effect of presowing treatment of seeds with insecticides on nodulating ability, nitrate reductase activity and plastid pigments content of lucerne. In: Medicago sativa, L. (Ed.), Agron Res., vol. 5, pp. 87–92.

Vasileva, V., Ilieva, A., 2011. Chemical composition, nitrate reductase activity and plastid pigments content in lucerne under the influence of ammonium and nitrate form mineral nitrogen. Agron Res. 9 (1–2), 357–364.

Weijers, D., Jürgens, G., 2004. Funneling auxin action: specificity in signal transduction. Curr. Opin. Plant Biol. 7 (6), 687–693.

Willige, B.C., Isono, E., Richter, R., Zourelidou, M., Schwechheimer, C., 2011. Gibberellin regulates PIN-FORMED abundance and is required for auxin transport-dependent growth and development in *Arabidopsis thaliana*. Plant Cell 23 (6), 2184–2195. http://dx.doi.org/10.1105/tpc.111.086355.

Wodzicki, T.J., 2004. Auxin – agent communication processes over a cell functional differentiation of the plant. Post. Biol. Kom. 31, 44–53 (In Polish).

Zhao, Y., 2008. The role of local biosynthesis of auxin and cytokinin on plant development. Curr. Opin. Plant Biol. 11, 16–22.