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EVALUATION OF SELECTED CHARACTERISTICS IN INDUSTRIAL HEMP AFTER PHYTOHORMONAL TREATMENT

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Plant growth and development is significantly influenced by phytohormones – endogenous molecules present naturally in plants. The best known plant hormones are auxins and cytokinins. This study examined the possible effect of externally applied plant hormone analogues (growth regulators): 1-naphthyl acetic acid (NAA) and 6-benzyl aminopurine (BAP) on industrial fibre hemp (*Cannabis sativa* L., variety Bialobrzeskie). Plants were treated with three different concentrations of NAA (5, 10 and 20 mg/l) and three different concentrations of BAP (10, 25 and 50 mg/l). Morphological and physiological characteristics, such as apical dominance, shoot branching, fibre properties, and flavonoid content were evaluated. The chosen variety of hemp had a significant response to exogenous application of growth regulators, as has been observed with other plant species. Most notably, completely understood and controlled synthetic auxin tretament has a potential to increase the bark fibre yield of hemp.

Keywords: *Cannabis sativa* L., growth regulators, apical dominance, axillary branches, morphology and development, fiber.

INTRODUCTION

It is a well-known fact that growth and developmental processes of plants are regulated by endogenous molecules called phytohormones. They naturally occur in plants and act as signalling compounds at very low concentrations (Sauer *et al*., 2013). There are five main groups of plant hormones: auxins, cytokinins, gibberellins, abscisic acid, ethylene and other growth regulators such as brassinosteroids, jasmonic acid, strigolactones. Auxins and cytokinins were discovered first and are the best understood (Sudan *et al*., 2014). Auxins play a key role in the maintenance of apical dominance and acid growth, responsible for cell elongation. They are synthesised in shoot apices and young leaves and basipetally transported to the roots, since their main effects are rooting, stimulation, induction of tropic responses and inhibition of axillary buds outgrowth (Balla *et al*., 2011).

Cytokinins are involved in the regulation of meristem activity (Werner *et al*., 2001), determine plant shape, allowing plant to adjust to site conditions, to compete succesfully and to give adequate responses to the environment. Both auxin and cytokinin have been known for a long time to act either synergistically or antagonistically to control several significant developmental processes, such as the formation and maintenance of meristem (Su *et al*., 2011). Shoot branching is a major determinant of plant architecture and is highly regulated by both endogenous and environmental factors. Auxins and cytokinins have long been known to have an important role in controlling shoot branching (Umehara *et al*., 2008). Climate change and current situation in crop production leads to a need for identifying and using of new sustainable resources. Hemp (*Cannabis sativa* L.) is a renewable resource that can provide fibre, oil and biomass in a short time due to the plant reaching maturity in 135 days on an average (Weiblen *et al*., 2015). The stem of hemp supplies both cellulosic and woody fibres - the core is lignified, while the cortex harbours long cellulose-rich fibres, known as bast fibres (Guerriero *et al*., 2013). Certain characteristics of agricultural crops related to production can be improved via breeding selection, chemical treatments or genetic engineering. Exogenously applied growth regulators – chemical analogues to plant hormones can be used to influence the shoot branching and can cause greater vegetative biomass and seed production (Spitzer *et al*., 2011; Hozlar *et al*., 2014; Kumlay, 2014). The effects of various plant hormones on the architecture of hemp have not been well studied so far. Therefore, the aim of this study was to document the changes in morphological characteristics of an industrial hemp variety cultivated mainly for fibre and seeds following treatment with growth regulators.

MATERIALS AND METHODS

A synthetic auxin, 1-naphthyl acetic acid (NAA; ICN Biochemicals Inc., USA), was used for the auxin treatment. The cytokinin effect was examined using 6-benzyl

aminopurine (BAP; Erba Lachema Ltd., Czech Republic). Stock solutions of 200 mg/ml NAA and 300 mg/ml BAP were prepared by dissolving the solids in 10 ml of dimethylsulfoxide (DMSO; Sigma Aldrich Ltd., USA) with shaking and incubating in a water bath at 45 ºC until a homogeneous solution was obtained. The stock solutions were stored in the refrigerator at 4 ºC. Prior to application to the plants, the solution was diluted using distilled water to a final concentration of 5, 10 and 20 mg/l NAA and 10, 25 and 50 mg/l BAP. Polyoxyethylene sorbitan monolaurate (TWEEN; Erba Lachema Ltd., Czech Republic) was added as a surfactant into solution.

`Bialobrzeskie` is a cultivar of industrial hemp bred in Poland, and the content of the psychoactive *trans*-Δ^9-tetrahydrocannabinol (THC) in this plant does not exceed 0.16 % on an average (de Meijer *et al*., 1992). It was chosen partly in order to avoid the certain security limitations that would apply to the scientific work with *C. sativa* . in Czech republic, when THC content exceeds 0.3 %. In addition, this cultivar was readily available as a registered variety by the Central Institute for Supervising and Testing in Agriculture since 2008. Since this hemp variety has quite a high germination rate, plants were grown from seeds, and they were not obtained by cloning. Seeds provided by company SEMO Smržice Inc. (Czech Republic) were randomly placed into plastic trays with perlite (milled and extruded volcanic stones), watered and left to germinate under constant white light. After few days, germinated seedlings were transplanted into a common horticultural substrate (AGRO CS Inc., Czech Republic), properly watered and grown in the cultivation trays, till they were mature enough. Then they were moved into a greenhouse where the experiment was about to take place, and finally transplanted into 10 litre plastic pots with the same substrate. Plants were arranged into seven groups – the control group, three groups to be treated with different concentration of NAA and three groups to be treated with BAP. There were ten plants in every group to ensure an adequate degree of statistical reliability. The control group did not receive any special chemical treatment, other than standard procedures, such as fertiliser and insecticide used for all the plants in the greenhouse. As soon as the plants reached the developmental stage of seventh stem node, the application of growth regulators began – four weeks (29 days) after sowing. Prepared aqueous solutions of NAA and BAP were sprayed on the plant leaves every two weeks, always on the same day of the week (totally four applications during the 49 days-long experimental period). At the end of the experiment (110 days after sowing), the entire fifth internode of every plant was sampled for fibre analysis. In addition, the apical parts of stems including flowers from each plant were collected to determine the total content of flavonoids present. The morphological characteristics that were measured and compared between groups were: average total stem length from the soil to the apex (plant height) and average length of the axillary branches. In order to obtain comparable data, axillary branches of only nine basal stem nodes were taken into consideration, as there was a tendency for irregular node development in the apical part of the plants. These characteristics were measured once a week, for a total of eight measurements, with the first measurement collected prior to the application of the growth regulators. Measurement was done manually with a flexible plastic or metal metric scale. Data collection was followed by a statistical analysis. Growth curves for stems and for axillary branches were done to visualise the rate of growth during time and statistical significance of different development between groups was verified by one-way ANOVA statistic. Possible differences were verified by "F" test ($P \leq 0.05$).

After the harvest, fibre content in the stem and flavonoid concentration in young tissues was measured. The fibre profile was evaluated using the method of Amaducci *et al*. (2008), that was applied to a 0.5 g stem portion at the fifth internode. After a treatment at 90 °C in 0.35% NaOH for 2 hours, bark and woody core tissues were separated. The bark portion was then treated with a 2% NaOH solution at 90 °C for an additional 2 hours. Both tissues types were rinsed with distilled water, dried in an oven (60 °C, 48 h) and finally weighed. The amount of fibres present was expressed as a percentage of the sample initial dry weight.

Flavonoid content was measured in the youngest leaves at the top of the plants using a multilabel plate reader (Perkin Elmer 1420 Multilabel Counter VICTOR3). Samples were ground in liquid nitrogen, and the flavonoids extracted overnight in 2 ml of methanol and finally precipitated by a centrifugation for 3 min and 10,000 g. A total 200 µl of the sample extract was then added to 100 µl of the probe DPBA (10 mg/ml of 2-aminoethyl diphenylborinate in ethanol) and then analysed for the fluorescence intensity.

The statistical analysis was performed on all data by the programme STATISTICA 10 (Statistica Tulsa, Oklahoma USA) and reported at the 0.05 level of significance. The significant statistical differences between means were established by LSD test ($P \leq 0.05$). Statistical models (ANOVA and mean separation by LSD test) were executed using the General Linear Model (GLM) procedures of the programme STATISTICA 10.

RESULTS

The effect of phytohormonal treatments applied at four different developmental stages was assessed for hemp plants, grown in a greenhouse. For the sake of simplicity, the sampling dates were related to a period of 49 days using as starting point the time of the first hormonal treatment, which occurred after 29 days after sowing (d.a.s.), while the harvest occurred at 110 d.a.s.

The NAA treatments generally influenced hemp development to a greater extent compared to BAP treatments. Auxins were

able to non-significantly inhibit the average plant height (Fig. 1) over a 49-day period in which the plants were exposed to growth regulators. Treatment with BAP did not affect the total height of the plants as they grew similarly with the control group (data not shown).

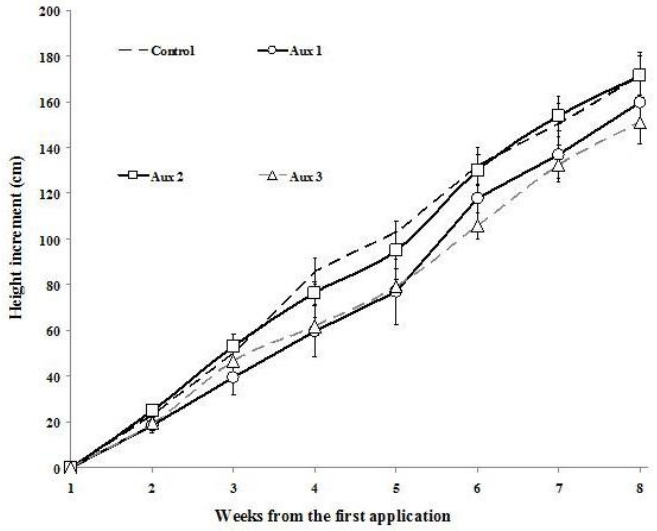

Figure 1. Average growth rate of stems in auxin-treated groups compared to control group.

Legend: Control = group with no treatment, Aux 1 = 5 mg/l NAA, Aux 2 = 10 mg/l NAA, Aux 3 = 20 mg/l NAA.

During the same period, both growth regulators stimulated axillary branch growth, which exhibited a continuous increase during time (Fig. 2), while the control group exhibited lesser development of the lateral branches.

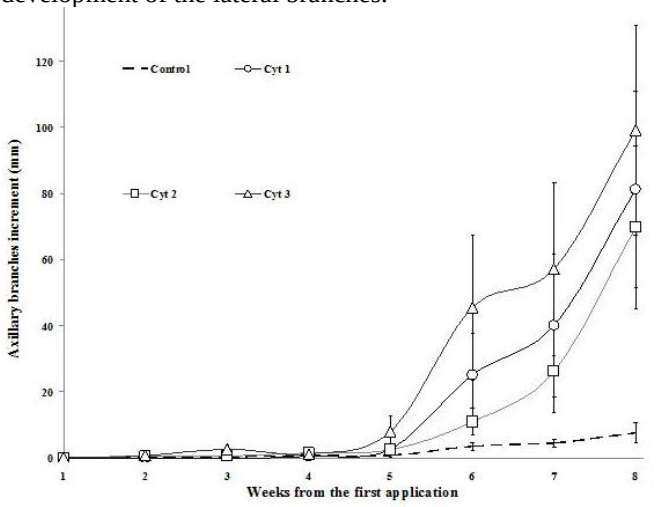

Figure 2. Growth curves for the axillary branches in all cytokinin-treated groups and control group.

Legend: Control = group with no treatment, Cyt 1 = 10 mg/l BAP, Cyt 2 = 25 mg/l BAP, Cyt 3 = 50 mg/l BAP.

A strongly significant statistical difference was found in the case of highest cytokinin dose at the sixth week of measurement ($p = 0.018$). Other cytokinin-treated groups, as well as intermediate dose of auxin showed significant increase in lateral branching ($p \leq 0.05$) during the last few weeks of the experiment. In case of auxin, significant difference in comparison with control group ($p = 0.047$) initially showed at the lowest dosage. During the last week of the experiment, significant differences between auxin-treated groups and untreated control group were already present at all concentrations used (Fig. 3).

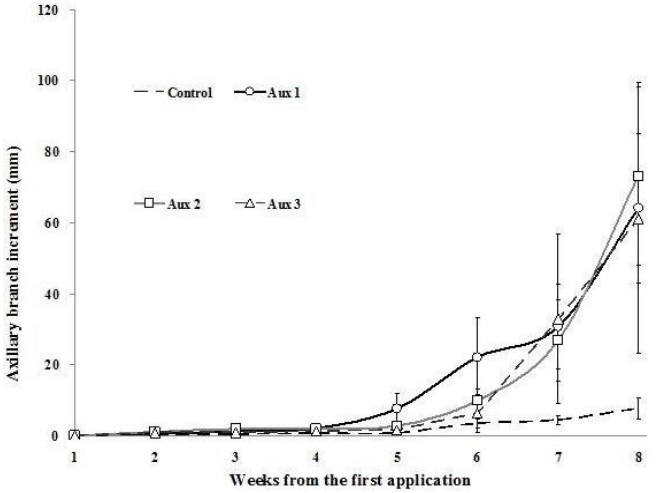

Figure 3. Growth curves for the axillary branches in all auxin-treated groups and control group.

Legend: Control = group with no treatment, Aux 1 = 5 mg/l NAA, Aux 2 = 10 mg/l NAA, Aux 3 = 20 mg/l NAA.

The NAA-treatments induced a highly significant ($p = 9\times10^{-5}$) dose-dependent stem enlargement in comparison to untreated control group. Cytokinin, however, was completely ineffective (Fig. 4).

Surely the most intriguing effect yielded by the NAA treatments on hemp plants was related to the modulation on secondary fibre distribution. The ratio between woody core fibres (xylem associated) and bark fibres was clearly very significantly modified by the auxin treatments ($p = 0.003$). The groups with the lowest and highest dose of NAA had an increased ratio of outer bark fibre when compared to the control group: 3.69 % increase for 5 mg/l and 2.63 % for 20 mg/l dosing on an average (Fig. 5). Again, the effect was not present in cytokinin-treated plants (results not shown).

Growth regulators were also assayed for the capacity to regulate secondary metabolism and in particular for their modulation in respect of the flavonoid synthesis. Both auxin-treated and cytokinin-treated plants displayed a similar pattern of flavonoid biosynthesis modulation, where the lower dose induced ahigher flavonoid content in hemp apical leaves, while at increasing hormone doses these secondary metabolites progressively declined (Fig. 6). However, ANOVA of these data did not show any statistically significant difference between groups exposed to growth regulators and a control group.

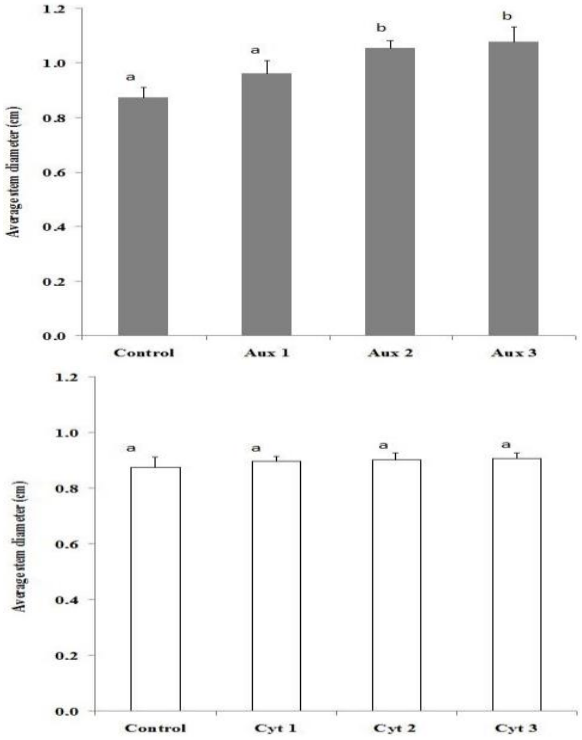

Figure 4. Changes in stem diameter at fifth internode between auxin-treated groups (picture on the left) and cytokinin-treated groups (picture on the right).

Legend: Control = group with no treatment, Aux 1 = 5 mg/l NAA, Aux 2 = 10 mg/l NAA, Aux 3 = 20 mg/l NAA, Cyt 1 = 10 mg/l BAP, Cyt 2 = 25 mg/l BAP, Cyt 3 = 50 mg/l BAP. Letters above the columns represent statistically significant differences in stem diameter of hemp plants.

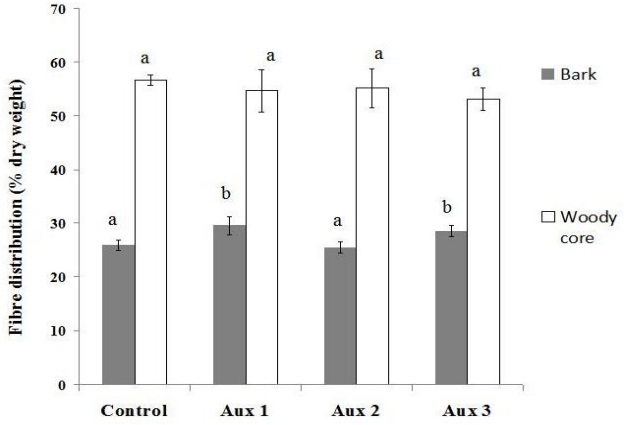

Figure 5. Percentual ratio of bark fibre to woody core fibre at the fifth internode of stems for all three auxin-treated groups compared to control group.

Legend: Control = group with no treatment, Aux 1 = 5 mg/l NAA, Aux 2 = 10 mg/l NAA, Aux 3 = 20 mg/l NAA. Letters above the columns represent statistically significant differences in fibre percentage within the bark and woody core of hemp stems.

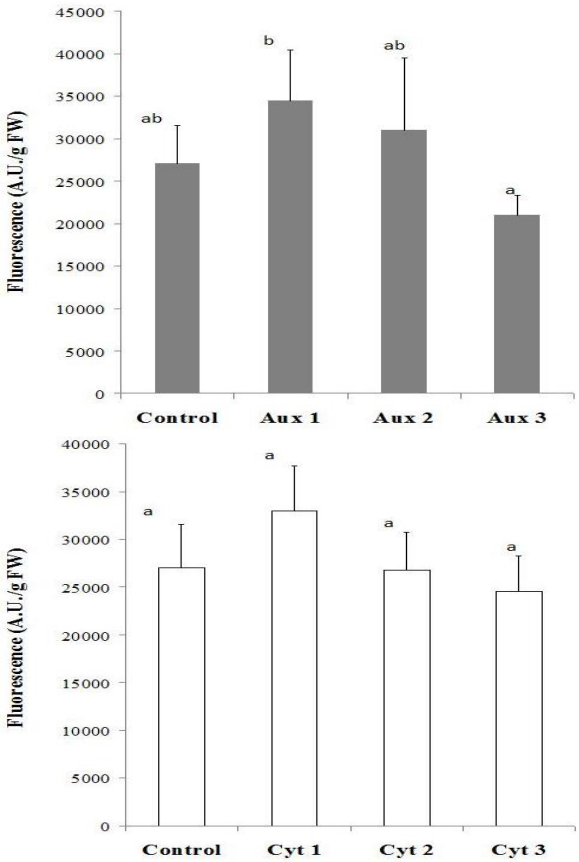

Figure 6. Flavonoid content in apical stem portion of hemp plant after treatment with auxins (picture on the left) and cytokinins (picture on the right) at different doses.

Legend: Control = no treatment, Aux 1 = 5 mg/l NAA, Aux 2 = 10 mg/l NAA, Aux 3 = 20 mg/l NAA, Cyt 1 = 10 mg/l BAP, Cyt 2 = 25 mg/l BAP, Cyt 3 = 50 mg/l BAP. Letters above the columns represent statistically significant differences in flavonoid content.

DISCUSSION

The results for the cytokinin-treated groups were consistent with previously published studies. Concentration dependent significant increases in axillary branches length due to cytokinin treatments was observed in previous studies done with legumes (Sachs and Thimann, 1967; Pillay and Railton, 1983; Li and Bangreth, 2003). Exogenous application of cytokinin has been shown to have no effect on modifying evaluated plant morphology variables in previous studies on soybean (Leite *et al*., 2003; Rasheed *et al*., 2017).

Results for auxin-treated groups are actually interesting, because it is widely known that auxins promote stem elongation and maintain the apical dominance (assuming that auxin treatment should have led to taller plants on an average) and in such features they are clearly distinguishable from cytokinins (Rayle and Cleland, 1992). Auxin plant groups

exhibited a decreased average height (stem length) when compared to the control (Fig. 1). The inhibitory effect in term of plant height and significantly promotive action in terms of axillary branch length (Fig. 2) might be explained by both an over-optimal NAA concentration and unbalance of hormonal endogenous equilibrium. Previous studies have shown that high auxin concentrations are inductive for ethylene synthesis and the release of this gas could be responsible for the decrease of stem elongation (Morgan and Hall, 1962; Hansen and Grossmann, 2000). In many plant species, the inhibition of shoot branching caused by exogenous auxin treatment (lanolin paste containing auxin) has been described (Thimann and Skoog, 1934; Morris, 1977), but also some exceptions have been recorded (Cline, 1996). Of course, the form of application chosen in this experiment (spraying the solution on leaves of hemp) can make a difference. Other factor could be represented by auxin action that is effective through polar transport as well. In hemp, synthetic auxin NAA could be interacting with a different set of receptors than the most common and natural auxin - indole 3-acetic acid (IAA). Similar observations were made in previous studies on kidney bean and tobacco (Smulders *et al*., 1990; Morris *et al*., 2005). In addition, NAA effect is not dose dependent since the group affected by intermediate dose (10 mg/l) did not induce any appreciable difference compared to the control group (Fig. 1., Fig. 2.) This behaviour seemed to be conflicting with results obtained by the analysis of stem diameter growth (Fig. 4), measured at fifth internode on hemp stem collected at harvest time. Although it is not clear why a directly proportional morphological changes due to the NAA dosage are present only in the case of stem diameter and no other variables, one major screening study concluded that the majority of auxin-induced dose responses are subsensitive, meaning that plant development is regulated by changes in the sensitivity to plant growth substances (Nissen, 1985).

The data for stem diameter might be explained by the fact that hemp has both primary and secondary development, despite the herbaceous growth. Due to this developmental feature shared with other fibre plants, e.g. flax, in a mature plant it is possible to distinguish three different anatomical portions. The apical part of the stem is characterised by primary growth only, while the first basal nodes exhibited a secondary development and finally the intermediate zone where both the anatomical structures are detectable (Snegireva *et al*., 2015). Furthermore, the intrusive fibre elongation in the phloem, a process associated to primary development, is temporally distinct from secondary cell wall thickening (Gorshkova *et al*., 2003). A possible explanation of the auxin effects relies in the observation that the primary growth is more sensitive and responds to a low NAA treatment. Conversely, they are able to modulate in a dose-dependent manner only secondary growth, where stem diameter is a marker, probably because this is a phenomenon requiring high-dose NAA. It is noteworthy that the stem diameter was measured at the fifth node, in the most differentiated portion of the stem. In the case of plant height and axillary branch growth, markers of primary growth, low concentration (5 mg/l) was already sufficient to provide active stem elongation. Higher amounts of auxin abolished and restored (10 mg/l and 20 mg/l, respectively) the hormonal effect. This biphasic behaviour of the response might depend on different tuning of the ratio with other endogenous hormones, due to treatment with concentrations of exogenous auxins exceeding the minimum effective dose.

The evidence for secondary fibre distribution has an interesting technological implication, because bark fibres are known to have the highest quality and are more appreciated, since they exhibit a high content of crystalline cellulose (Sankari, 2000; Behr *et al*., 2016). Hypothetically, certain doses of auxin may trigger the increased elongation in stems of hemp via acidic effect on the cell wall (primary growth), while in other doses the effect of auxin is visible in the increased investment for the development of bark mass (secondary growth) because of the relationship to ethylene, as it was described in several tree species (Savidge, 1988). This idea may be supported by a possible correlation with the results of average stem length, where the same pattern is present, although non-significant - the intermediate NAA dosing (10 mg/l) showed practically no difference in stem length from the control group and at the same time it was the only dosing without a significant effect on bark fibre distribution. Increase of fibre yield due to auxin treatment was found under *in vitro* conditions also in one study with cotton (Chavan *et al*., 2014).

Flavonoids are involved in auxin protection because they prevent degradation activity due to peroxidase activities (Savitsky *et al*., 1999). Peroxidases are responsible for auxin decrease during the last stages of fibre cell wall formation and are more present in hemp male phenotype (Truță *et al*., 2002). The pattern shared by both hormonal treatments seems to be a generic effect on flavonoid metabolism, mainly caused by the induction of growth. An increased carbohydrate request induced by the growth regulators could have competed with the phenylpropanoid pathway, which is equally dependent on sugar supply. In addition, IAA application at high concentration to iceberg lettuce (*Lactuca sativa* L.) was reported to severely decrease phenylalanine ammonialyase activity and flavonoid content (Dangyang and Saltveit, 1988). The interaction between growth regulators and secondary metabolite biosynthesis deserves additional research. De Pauw *et al.* (2007) found an increase of transcripts associated to phenyl propanoid metabolism in hemp phloematic tissues, in particular where secondary cell wall thickening and lignification were occurring.

Conclusions**:** *Cannabis sativa*. seems to have a standard response to exogenous treatment by cytokinins, comparable to the one observed in pea and fava bean for example.

Response to synthetic auxins has had discrepancies and should be studied further, to fully understand the possible synergic effect on hemp fibre synthesis exerted by auxins together with other growth regulators, namely cytokinins and gibberellins. This would be achieved for example by studying the behaviour of phytohormones in vascular structures of hemp by immunochemical methods. Some care needs to be devoted to growth regulator doses, which probably need to be more finely tuned, since they seemed to be somehow in an over-optimal concentration. However, synthetic auxin treatment was found to have some promising potential in increasing the bark fibre yield. In any case, it has to be stressed that the experimental plan was performed in a greenhouse, where hemp plants could maintain stomata open due to moisture saturated atmosphere. The hormonal treatment, therefore, might have been magnified by this environmental condition and this would not be the case if the auxins would be applicated to open field cultivation. Together, these results are promising in terms of improving fibre quality production from hemp cultivation, mainly the positive effect on the bark fibre synthesis exerted by auxin, as already demonstrated in other fibre crops.

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