WASTES FROM FORESTRY AND ENERGY INDUSTRIES AS POTENTIAL BIOREGULATORS IN SOYBEAN (*GLYCINE MAX* L.) PLANTS

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This article is dedicated to the anniversary of 50 years since the founding of the Cellulose Chemistry and Technology journal. On this anniversary, we hope that both the editorial board members and collaborators will contribute further to increasing the quality and prestige of Cellulose Chemistry and Technology.

Polyphenols are the only group of phytochemicals present in all plants. Their activity is based upon functional groups capable of accepting a free radical's negative charge. In our experiments, we have evaluated the effect of spruce bark polyphenolic extract (SBPE) and deuterium depleted water (DDW) as soybean plant bioregulators. After aqueous extraction and quantification, as well as identification of polyphenolic compounds by HPLC methods, we applied the extracts obtained, together with DDW,in the growth medium of soybean plants. The biological activity of SBPE and DDW was assessed by determining the germination energy and capacity, biomass accumulation, assimilatory pigment accumulation, photosynthesis activity, substomatal cavity CO_2 concentration, transpiration and respiration rate, as well as photosynthetically active radiation (P.A.R.) of the leaf surface. The obtained results showed that the highest stimulating effect on germination energy and capacity was registered in the presence of DDW in combination with spruce bark polyphenolic extract (SBPE). Soybean plants treated with SBPE reached the highest amount of biomass, compared to the other experimental variants. DDW, in combination with SBPE, enhanced the assimilatory pigment accumulation in soybean plants. The photosynthesis activity, transpiration and respiratory processes were considerably intensified, especially in the presence of SBPE.

Keywords: bioregulators, deuterium depleted water, polyphenols, spruce bark

INTRODUCTION

Soybean (*Glycine max* L.) is one of the most important crops worldwide, as it is an important source of oil, protein and secondary metabolites. Soybean yields have increased substantially throughout the past century, with yield gains widely attributed to genetic advances and improved cultivation techniques, as well as to advances in farming technology and practice.[1]

Tree barkis a by-product of log processing and has the primary function of storage and translocation of assimilates, besides that of physical and physiological defense of the living tree. It can contain large amounts of extracts, contributing against predators protection and it can be recovered from log processing.[2,3] One of the most common and abundant groups of these extractives are polyphenols, which can be found in every vegetable cell, protecting it against biotic and abiotic stress owing to their antioxidant and other related properties.[3] They are essential for plant growth and may exhibit a wide range of biological properties, such as anti-allergenic, anti-inflammatory, anti-microbial, antiviral, antioxidant, cardioprotective, vasodilatory effects in relationship to human health.[3,4] Recent studies have shown that these polyphenols can be extracted from bark,[4-9] as well

as from other tissues (e.g. knots, cones),[8,10] and could be utilized in the future for nutrition supplements,[5] wood preservatives[6]or food antioxidants.[5]

In the present study, spruce bark was chosen as raw material for separating polyphenols. The choice was based on resource availability, as spruce bark is a waste generated in large amounts from wood processing. Taking into account the results obtained regarding the total polyphenols content, and the content of tannins, anthocyanins, flavones, flavonols and others, it could be concluded that spruce bark represents an important source of phenolic compounds, such as gallic acid, vanillic acid or catechine.[11,12]The application of polyphenols as a biostimulator for plant growth is very topical in today's plant science and their properties have been tested on a large group of plants, such as sunflower, bean, oat, rape, maize and tomatoes, as described previously.[11-16]

Recent research has shown that spruce bark extract and DDW have a great influence on the growth and development of plants, namely in developing cells and tissues, and the changes that occur under normal water conditions lead to significant modifications in the fundamental biosynthesis processes of the cells.[11,12] DDW and spruce bark extracts enhance the assimilatory pigment accumulation in sunflower plants, with some differences, determined by varying polyphenolic extract concentrations. The supplementation of the growth medium with polyphenolic extract (SB1 and DDW+SB) determines an increase in the amount of catechine in leaves, thus increasing the defense capability and therefore improving plant resistance, growth and development.[12-14]

So far, we have observed the influence of SBPE and DDW on soybean seedlings in germination tests or in vegetative pots. We found that spruce bark aqueous extract in combination with deuterium depleted water stimulates the elongation of all vegetative organs and the accumulation of biomass for soybean seedlings.[14]In the present study, we assessed the influence of SBPE and DDW on soybean plants grown in the field.

The aim of this study was to evaluate the effect of deuterium depleted water and spruce bark polyphenolic extract as soybean plant bioregulators. In addition, the combination of deuterium depleted water with polyphenol extract could be used in order to obtain information about their role in plant development.

EXPERIMENTAL

Plant materials

Spruce bark (*Picea abies* L.), a major waste product from the forestry and paper and pulp industries, was purchased from the Alpine LTD Timber Company, Vatra Dornei, Romania. The vegetal material was randomly collected, while the species were identified and authenticated. After drying (at normal temperature and aeration) and grinding, the vegetal material was extracted using distilled water. 10 g (SB1) or 5 g (SB2) of ground dried spruce bark with 0.5-1 mm particle size were extracted three times, using 125 mL distilled water on a water bath, for 45 min at 80°C,and the extracts were cumulated to a final volume of 500 mL.[11-13,17]

Deuterium depleted water (DDW)or light water is a distilled microbiologically pure water, with an isotopic concentration of 25 ppm, obtained by isotopic distillation, in vacuum, of natural water with an isotopic concentration of 145 ppm D/(D + H).[18]

Soybean seeds were purchased from a seed distribution company (UnisemImpex SRL, Romania).

Experimental assays

The experiments were performed in the field of "Anastasie Fatu" Botanical Garden of Iasi.

The aqueous extract was characterized in terms of total polyphenolic content using the Folin Ciocalteu method. The extracts were also characterized from the point of view of total content of tannins, flavonoids, flavonols, and antocyanins.These results were published in our previous work.[11-13]

The experiment was preceded by numerous tests of the same variants in greenhouse conditions by testing germination or plants growing in vegetative pots.[14]The field experiment was performed over a period of 120 days from April to August, during two consecutive years (2010 and 2011), and was carried out in triplicates. The average value was reported. The following test solutions were used: tap water(control), deuterium depleted water (DDW), deuterium depleted water in combination 1:1 with spruce bark extract with 191 mg GAE/L (DDW+SB), spruce bark extract with 130 mg GAE/L (SB1) and spruce bark extract with 191 mg GAE/L (SB2).

The application of test solutions, in order to allow the compounds to be absorbed, was done periodically, starting with the appearance of the first leaf. During the vegetative growth and development stage of the soybean plants, the applications were carried out at the baseline and at the foliar level by spray irrigation. The amount of the applied solution was 10 mL/plant/application.

To determine the elongation and biomass accumulation, ten plants from each experimental variant were separated into root, stems and leaves, 120 days after seedling. They were first dried at 65 °C for enzyme inactivation and then at room temperature, and weighed. The results obtained are the average of the measurements of ten samples.

The assimilation rate (A,μmol $CO_2m^{-2}s^{-1}$), substomatal cavity CO_2concentration (Ci,μmol

$CO_2 mol^{-1}$), transpiration rate (E,µmol H_2O $m^{-2}s^{-1}$) and P.A.R. leaf surface (Qleaf,µmolm^{-2} s^{-1})were determined with a LCi portable photosynthesis system (ADCB Bioscientific Ltd., Hoddesdon 2004, UK). Measurements were performed from the youngest fully expanded leaf of each plant. Five measurements per plot were carried out from 11.00 to13.00 h, after 30 and 60 days of growth.

The concentrations of chlorophyll and carotenoid pigments were calculated using the specific coefficients suggested by Lichtenthaler and Wellburn.[19]

Statistical analysis

All the results are expressed as mean ± standard error, where $n = 3$. A comparison of the means was performed by the Fisher least significant difference (LSD) test ($p \leq 0.05$) after ANOVA analysis, using PAST 2.14program. Sampling and chemical analyses were performed in triplicate, in order to decrease the experimental errors and to increase the experimental reproducibility.

RESULTS AND DISCUSSION

Germination process of soybean seeds

The study on the influence of polyphenolic extracts on the seed germination process is part of a sequence of steps that need to be taken in order to assess the bioregulatory properties of the compounds studied.[20] Data analysis from literature sources provides numerous indications on the stimulating or inhibitory effect of polyphenolic compounds on the seed germination process.[12-14,21-25]

The obtained data indicate that the tested products have a stimulating effect on the germination energy and capacity, compared to the control (water). The experiment showed a high biological activity of spruce bark aqueous extract concentration with 130 mg GAE/L (SB1) and 96 mg GAE/L (DDW+SB) (Fig. 1). Analyzing the influence of SBPE on the germination process, it was observed that SB1 promotes the intensification of germination capacity by 17%, compared to the control.

Elongation of soybean plant (*Glycine max* L.) vegetative organs

In order to determine the growth differences in the length of the experimental variants for each vegetative organ, biometric techniques were applied. The effect of stimulation/inhibition was calculated in percentage, compared to the control. Fig. 2 shows that soybean plants responded positivelywhen they were treated with the polyphenolic extract obtained from spruce bark. At concentrations of 130 mg GAE/dm^3 (SB1) and 96 mg GAE/L (DDW+SB), the highest differences could be observed in the degree of stimulation, compared to the control. The increase in the length of the vegetative organs for variant SB1 was as follows: root – 46%, stem – 34%, leaves – 47%. In the case of variantSB2, the values were slightly lower.

DDW caused an evident stimulation increasing the length of the stem (21, 61%), compared to the value recorded in the control samples. The degree of the stimulation of length elongation for other vegetative organs was reduced.

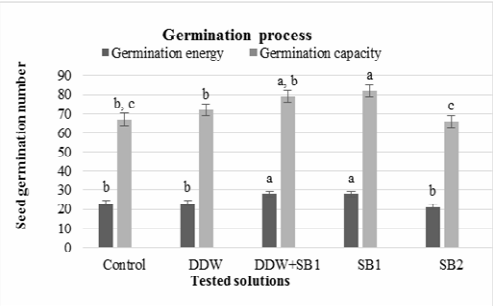

Figure 1: Influence of SB1 and SB2 aqueous extracts, in combination with DDW, on germination of soybean seeds. Bars show that the same letter is not significantly different at $p \leq 0.05$. Error bars represent the standard deviation of means (n = 3). (DDW – deuterium depleted water; DDW+SB – deuterium depleted water in combination (1:1) with spruce bark aqueous extract containing 191 mgGAE/dm^3; SB1 – spruce bark aqueous extract containing 130 mg GAE/dm^3; SB2 – spruce bark aqueous extract containing 191 mg GAE/dm^3)

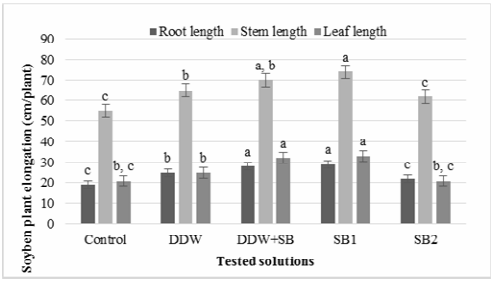

Figure 2: Influence of SB1 and SB2 aqueous extracts, in combination with DDW, on soybean plant length (The explanation to the symbols used is the same as in Fig. 1)

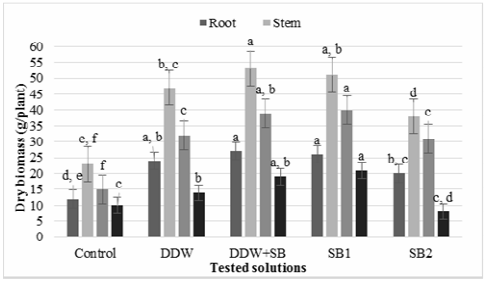

Figure 3: Influence of SB1 and SB2 aqueous extracts, in combination with DDW, on soybean dry biomass accumulation (The explanation to the symbols used is the same as in Fig. 1)

Biomass accumulation

The biomass accumulation data (Fig. 3) are correlated with the increase in length elongation (Fig. 2). These results underline once again the allelopathy effect of deuterium depleted water and spruce bark polyphenolic extract. The amount of biomass accumulated in the soybean plants was higher in all experimental variants, compared to the control (Fig. 3). Thus, a higher stimulation level is recorded by DDW+SB and SB1 variants, with a maximum value obtained in the case of the stem. A higher concentration of the extract (SB2) triggers a lower accumulation of biomass than in the case of SB1 and DDW+SB, but higher compared to the control. As shown in Fig. 3, the DDW causes an increase in the amount of biomass accumulated in all thevegetative organs and in seeds, as well as anincreased level of stimulation in thecase ofthe DDW+SB variant.

Physiological processes in soybean plant

It was observed that the photosynthesis activity was considerably improved in the soybean plants developed in the presence of all aqueous extracts, as determined after 30 and 60 days of growth. Analyzing the variation of the assimilation rate for the soybean plants in the presence of the tested solutions, it was noticed that the highest intensity was identified in the plants developed in the presence of the spruce bark extract (for SB1 by 21.72% and for DDW+SB by22.66% higher than the assimilation rate registered for the control). In addition, increases in the transpiration rate and substomatal CO_2 concentration for these variants were noted (Table 1). The high amount of biomass and the increase in the length of the vegetative organs may be correlated with the intensification of the assimilation rates. The photosynthesis process was considerably improved in the presence of all the natural products investigated, as assessed 30 and 60 days after sowing. After 60 days of growth, an increase in the assimilation rate, as well as in substomatalCO_2 and transpiration rate, in soybean plants was observed. This observation was made especially regarding the plants treated with DDW+SB and SB1 variants, where the obtained values are considerably higher than that of the control (Table 1). The high quantity of photo-synthetically active radiation can contribute to an increase of the photosynthesis rate (P.A.R.), transpiration rate and quantity of substomatalCO_2.

Analyzing the results obtained 30 days after sowing (Table 2), a general improvement of pigment assimilation was noted in the plants treated with the tested solutions. The highest stimulatory effect on pigment assimilation inthe plants was measured for the SB1 treated ones (Chl a concentration by 51.41% higher than the one determined for the control, Chl b concentration – by 47.62% and carotene content – by 97.77% higher than the values recorded for the control). Significantly increased values were registered also in the case of the spruce bark extract and deuterium depleted water (DDW+SB) treatment: higher by 47.16% for Chl a, 44.44% for Chl b and 113.33% for carotene pigment content, compared to the values for the control. The use of spruce bark extracts and deuterium depleted water enhances the biosynthesis of assimilatory pigment accumulation in soybean plants, and the differences can be correlated with the polyphenolic extract concentrations. The pigment concentration accumulated in soybean plants after 60 days of growth presents the same trend of evolution as that observed after 30 days, but the value registered after 60 days presents a higher stimulatory effect. All tested solutions exhibited an increased content of assimilatory pigments.

Table 1
Physiological indices for soybean plants grown under treatment with SBPE and DDW

Sample	Time of measurement, days	Substomatal CO_2, vpm	Assimilation rate, µmol CO_2 $m^{-2}s^1$	Transpiration rate, mmol µmol H_2O m^{-2} s^1	P.A.R. incident on leaf surface, µmol CO_2 $m^{-2}s^1$
Control	30	221±2.14^{e}	17±0.12^{d}	7.1±0.12^{e}	1423±12.14^{f}
DDW		232±2.11^{d}	21±0.11^{b,c}	12.3±0.15^{b}	1546±14.31^{d,e}
DDW+SB		271±2.18^{a}	30±0.18^{a}	14.3±0.09^{a}	1632±10.91^{c}
SB1		269±2.21^{a}	31±0.12^{a}	12.5±0.15^{b}	1734±11.13^{a}
SB2		214±2.11^{f}	22±0.19^{b,c}	11.8±0.12^{b,c}	1654±21.11^{b,c}
Control	60	245±3.11^{f,g}	18±0.13^{d,e}	8.7±0.14^{f,g}	943±11.24^{f,g}
DDW		263±2.12^{e,f}	24±0.16^{b,c}	13.9±0.11^{b,c}	1213±11.42^{d,e}
DDW+SB		312±2.01^{b}	32±0.11^{a,b}	18.3±0.12^{a}	1432±12.01^{b}
SB1		323±1.68^{a}	35±0.15^{a}	13.9±0.09^{b,c}	1543±9.19^{a}
SB2		251±2.13^{f}	27±0.12^{b}	12.1±0.11^{d,e}	1291±14.04^{c,d}

Superscript letters indicate significant differences ($p < 0.05$). Error bars indicate standard error of the mean (n = 3);(DDW – deuterium depleted water; DDW+SB – deuterium depleted water in combination (1:1) with spruce bark aqueous extract containing 191 mgGAE/dm^3; SB1 – spruce bark aqueous extract containing 130 mg GAE/dm^3; SB2 – spruce bark aqueous extract containing 191 mg GAE/dm^3)

Table 2
Photosynthetic pigment content (µg/g) for soybean plants grown under treatment with SBPE and DDW

Sample	Time of measurement, days	Chl a, µg/g	Chl b, µg/g	Carotene, µg/g	Chla+b	Chl a/b
Control	30	212±2.54^{e}	63±0.52^{d}	45±0.81^{e,f}	275	3.36
DDW		232±2.01^{d,e}	78±0.61^{b,c}	68±0.73^{d}	310	2.97
DDW+SB		312±2.03^{a,b}	91±0.98^{a}	96±0.86^{a}	403	3.42
SB1		321±2.12^{a}	93±0.52^{a}	89±0.61^{b}	414	3.45
SB2		243±2.32^{c,d,e}	81±0.69^{b,c}	76±0.72^{c}	324	3.01
Control	60	211±2.11^{f,g}	71±0.74^{e}	56±0.83^{e,f}	282	2.97
DDW		263±1.52^{e,f}	83±0.76^{b,c}	83±0.49^{c}	346	3.16
DDW+SB		321±1.31^{b}	99±0.96^{a,b}	99±0.87^{a}	420	3.24
SB1		343±1.98^{a}	101±0.85^{a}	93±0.63^{a,b}	444	3.39
SB2		276±2.05^{d,e}	78±0.82^{d}	78±0.78^{d}	354	3.53

Superscript letters indicate significant differences ($p < 0.05$). Error bars indicate standard error of the mean (n = 3);(DDW – deuterium depleted water; DDW+SB – deuterium depleted water in combination (1:1) with spruce bark aqueous extract containing 191 mgGAE/dm^3; SB1 – spruce bark aqueous extract containing 130 mg GAE/dm^3; SB2 – spruce bark aqueous extract containing 191 mg GAE/dm^3)

The ability to stimulate or inhibit plant growth and development was closely correlated with the concentrations of the polyphenolic compounds applied. Thus, in our experiments, the presence of these compounds in low concentrations (130 mg GAE/dm^3) had a beneficial effect on the development of plants.

CONCLUSION

In the present research work, we demonstrated the bioregulating influence of SBPE and DDW in the growth of soybean plants. From the obtained data, we can conclude that polyphenolic products and deuterium depleted water can act as allelochemicals in the case of soybean cultivation, thus confirming the results of other experiments carried out on sunflower and corn. Soybean plants treated with SB1 reached the highest amount of biomass, compared with the other experimental variants. At a higher concentration of the spruce bark extract, the stimulating effect on soybean plant biomass accumulation was reduced. The photosynthesis activity and transpiration process were considerably intensified, especially under the treatment with the spruce bark aqueous extract (SB1) and DDW+SB. In addition, DDW and spruce

bark extracts enhance the assimilatory pigment accumulation in soybean plants, with some differences, caused by varying polyphenolic extract concentrations.

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