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Effect of Plant Nutrients on Antiradical Activity of *In Vitro* Cultivated Broccoli (*Brassica oleracea* L. var. *italica* Plenk.)

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Abstract: Environmental conditions may have impact on plant metabolism, especially on secondary metabolism. As a result of different stress circumstance, plants have developed different protective mechanisms and major one is production of secondary metabolites. Plant growth conditions could be controlled and modified in *in vitro* plant culture, which usually results in higher or lower contents of secondary metabolites. We have established a rapid protocol for *in vitro* germination and cultivation of *Brassica oleracea* L. var. *italica* Plank. Three, ten, twenty and thirty days old seedlings, cultivated on three different Murashige-Skoog (MS) media, as well as two types of spontaneously induced calli were used for extraction. Ethanolic plant extracts were tested for their antioxidative potential using 2,2'-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging method. Extracts from three days old seedling demonstrated the highest antioxidative potential. On the other hand, extract of broccoli seedlings cultivated on basal MS medium have shown prooxidative properties that can be contribute to prooxidative properties of some unknown component in the presence of free transition metal ions, the type of oxidizable substrate in use, as well as to the biological environment in which they act.

INTRODUCTION

Oxidative stress, caused by reactive oxygen species (ROS), is one of the main cause of many pathological conditions of the human organism. At the molecular level, ROS induce several types of DNA damage (Halliwell and Aruoma, 1993). There are several ways to protect the organism from the harmful effects of "active" metabolic oxygen and one of them include antioxidant components. Antioxidant compounds represent a group of protective agents and defense mechanisms, whose role is regulation of cell redox state. Although almost all organisms possess antioxidant mechanisms to prevent and repair oxidative damage, often these endogenous mechanisms are insufficient for the prevention of oxidative stress and the diet should be supplemented with additional quantities of exogenous antioxidants. In this connection it is noteworthy that many plant species contain various components that possess antioxidant activity, such as vitamins C and E, β-carotene and polyphenols (Diplock, Charleux, Crozier-Willi *et al.*, 1998). For all these reasons, plant species are very important in the human diet. Epidemiological studies point out that consuming large amounts of antioxidants from fruits and vegetables could prevent carcinogenesis in many human and animal tissues (Bonnesen, Eggleston, Hayes, 2001). Also it has been shown that aqueous and ethanolic extracts of *Brassica oleracea* L. var. *italica* Plenk. (Brassicaceae), have strong antioxidant properties (Čakar, Parić, Maksimović *et al.*, 2011), and are very effective in scavenging superoxide anion and hydrogen peroxide (Gülcini, Sat, Bezdemir, *et al.*, 2004; Eberhardt, Kobira, Keck *et al.*, 2005). Elicitation of plant tissues in *in vitro* conditions is a valuable technique to study secondary metabolite production. Elicitation is useful technique for

induced production of secondary metabolites and therefore plant growth and development will be suppressed (primary metabolism-growth and development is decreased when secondary metabolism is induced). Secondary, metabolites are known to have a prominent antioxidant activity. Consequently, modification of plant growth conditions could affect antioxidative capacity of plant extracts.

The objective of our study was to investigate the influence of plant growth regulators on morphogenesis and antiradical activity of broccoli extracts. Concerning this, particular highlights to the influence of the type and concentration of plant growth regulators on plant development and antioxidative activity should be made.

EXPERIMENTAL

In vitro culture of broccoli

To establish *in vitro* tissue culture of broccoli, commercially purchased seed (*SEMENTI Franchi SpA Grassobbio-BG–Italy*) has been used. Sterilization of seeds was carried out in aseptic conditions, in absolute alcohol for one minute and 20 minutes in 15% sodium hypochlorite. Seeds were then rinsed in sterile distilled water and placed on MS (Murashige and Skoog (Murashige and Skoog, 1962) medium supplemented with appropriate combinations and concentrations of plant growth regulators. Three types of MS media was used. The first MS media used was basal MS medium (WH). Second (H) was MS medium supplemented with 0.1 mg/L 6-benzylaminopurine (BAP) and 0.1 mg/L indol butyric acid (IBA). The third one (3H) was MS medium with 0.5 mgL BAP, 0.2 mg/L IBA and 0.1 mg/L gibberellic acid (GA_3). Seedlings collected at third, tenth, 20th and 30th day, cultivated on three MS media, were utilized for extraction. Additionally, spontaneously formed calli on H and 3H media were also used for extraction.

Prepartion of extracts

Plant material (2 g) was subjected separately to Soxhlet extraction with 96% ethanol. Each extracts were filtered and concentrated in rotary evaporator at approximately 40°C. Finally, extracts were sterilized filtrating throughout syringe filters with 0.20 μm and stored at +4°C.

Antioxidative activity measuring

Antioxidative activity of extracts was determined using slightly modified 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) scavening radical method (Kulisic, Radonic, Katalinic *et al.*, 2003). Extracts were diluted to the three different concentrations (1:1, 1:3, 1:5) and aliquot of each extract solutions (100 μL) was mixed with 3 mL of 0.001% $DPPH^{\bullet}$ dissolved in absolute ethanol. The reaction of scavenging $DPPH^{\bullet}$ was carried out at room temperature in the dark for 30 min. Blank was a 100% ethanol and thymol was used as a positive control. The radical-scavenging activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to Yen and Duh (1994).

$$\%Inhibition = \frac{(A_0 - A_t)}{A_0} \cdot 100$$

where A_0 is absorbance of the control at t = 0 min, A_t absorbance of the antioxidant at t = 30 min.

Statistical analysis

Experimental results were represented as the mean value of the three replicates with standard deviation (SD). Shapiro-Wilk test showed that data were not normally distributed, so Kruskal-Wallis test was used for testing differences between means of antioxidative activity of broccoli extracts cultivated at different media types and at different growth stage. One sample *t*-test was used for testing differences between means of antioxidative activity of broccoli extracts and positive control. Differences were considered significant at $p < 0.05$. For all analysis MedCalc® Version 10.4.0.0 software was used.

RESULTS AND DISCUSSION

In vitro culture of broccoli

Germination of *B. oleracea* var. *italica* shoots was achieved in all three types of MS media. After 20 days some differences in plant growth and development of *in vitro* seedlings have been observed, which could be correlated with different combinations and concentrations of exogenously added phytohormones. Explants cultivated on the WH and 3H media were significantly longer than shoots cultivated on H medium (Figure 1). A large number of lateral shoots were recorded on the explants cultivated at all three media. Many authors emphasize the influence of proper cytokinin/auxin combination on the formation of adventitious shoots in *in vitro* conditions (Lazzeri and Dunwell, 1986; Msikita and Skirvin, 1989). As an optimal combination for the lateral shoot formation in broccoli, authors indicate combination of 0.1 mg/L BAP and 0.1 IBA mg/L. This combination was favourable for lateral branching in our study also. Calli were induced on plantlets roots cultivated at the media with the addition of growth regulators (H and 3H). It is well known that the presence of cytokinins and auxins in the medium often caused callus formation (Widiyanto and Erytrina, 2001). Rhizogenesis was successfully obtained when shoots were cultivated in all three types of MS media. The roots were visible after twenty days.

Figure 1. Effect of different combinations and concentrations of plant growth regulators on growth of broccoli; WH – MS medium without hormones, H – MS medium with 0.1 mg/L BAP and 0.1 IBA mg/L and 3H – MS medium with 0.5 mgL BAP, 0.2 mg/L IBA and 0.1 mg/L GA_3.

Antiradical activity of broccoli

It is well known that plant growth regulators affect growth and development, and therefore they have an impact on the production of secondary metabolites. Research studies suggest that the addition of auxins in medium could increase the production of glucosinolates in broccoli, which have strong bioactive properties [Pasquali, Goddijn, De Waal *et al.*, 1992; Zhang, Li, Tang, 2005). Three days old broccoli sprouts exhibit the strongest antiradical activity regardless of the MS media type (Figure 2).

These extracts showed significantly stronger antiradical activity than thymol (t_{WH} = 6.315, p = 0.0032; t_H = 4.53, p = 0.011; t_{3H} = 5.364, p = 0.006). On the other hand, extracts of broccoli seedlings cultivated for ten days on H medium (428.57 mg/mL) and twenty days on H3 medium (434.48 mg/mL) showed very low antioxidant potential. Statistical analysis reported that the three-day-old seedlings shown significantly higher activity than plantlets cultivated for a longer period (H = 10.92, p = 0012). Antioxidant potential of broccoli extracts was published in several papers, and has shown that broccoli extracts have a strong potential for free radicals scavenging. Gülcini, Sat, Bezdemir, *et al.* (2004) examined the antioxidant properties of aqueous and ethanolic extracts from broccoli flowers. These authors reported that both types of extracts showed high antioxidant potential. Piao et al. (Piao, Kim, Yokozawa *et al*., 2005) isolated two active compounds (1.2-disinapoylgentiobiose and 1-sinapoyl-2-feruloylgentiobiose) from broccoli extracts. Our results showed that antiradical activity of both compounds is lower (5.18 mg/mL and 7.52 mg/mL, respectively) compared to antiradical activity of three days old broccoli obtained in our study. Antioxidant potential of broccoli may be directly related to the total amount of phenolics and flavonoids presented in the extracts (Dong-Jiann, Chun-Der, Hsien-Jung, *et al*., 2004; Moreno, Carvajal, Lopez-Berenguer, *et al*., 2006).

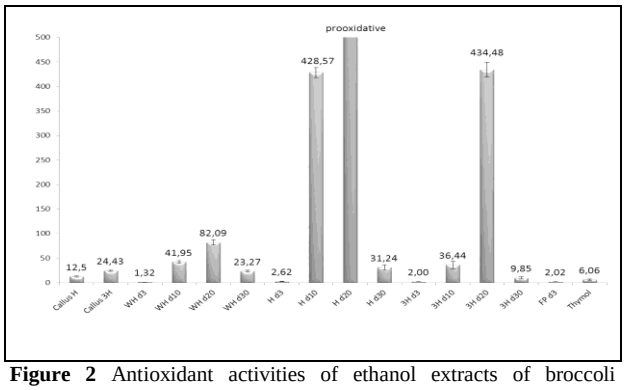

Figure 2 Antioxidant activities of ethanol extracts of broccoli cultivated at three different types of MS media.

In accordance with our results, it can be considered that the amount of phenolic content is higher in younger broccoli shoots. On the other hand, no statistically significant differences were recorded in the antioxidant potential of shoot extracts which origin from different types of MS media (H = 0.63, p = 0.83). Antiradical potential of broccoli extracts measured by DPPH method colud not be attributed to the various combinations of phytohormones. Ethanolic extract form 20 days old broccoli sprouts from H medium, showed prooxidative activity. Prooxidants increased the concentration of active oxygen and free radicals and encourage the growth of neoplastic cells. Hu, Zhang, Kitts (2000) suggest that the prooxidative activity of extracts can be due to the effects of plant phenolics in the presence of some transition metal ions. Similarly, Azuma, Ippoushi, Ito *et al.* (1999) showed that prooxidative properties of some extracts may be attributed to ascorbic acid, which in the presence of transition metal ions generates free radicals. Also the type of substrate used for oxidation, as well as the biological environment in which they act could alter oxidant/prooxidant activity of plant extracts (McGorum, Fry, Wallace *et al.*, 2000).

CONCLUSIONS

We have demonstrated in this study the effects of growth regulators on morphogenic response of broccoli cultivated *in vitro*. Appropriate combinations and concentrations of plant hormones result in higher yields of plant biomass, that is of great importance in plant mass propagation. Modification of growth conditions often results in changes of secondary metabolites production. In our work correlation between growth regulators and antiradical activity of extracts of broccoli has not been established. On the other hand, the radical DPPH$^{\bullet}$ test showed strong antiradical activity of three days old broccoli seedlings, regardless of the medium type. Presented results are preliminary in character, and further studies could be directed on the identification of specific bioactive compounds in broccoli extracts by HPLC-DAD-MS analyses.

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Summary/Sažetak

Uslovi spoljašnje sredine modificiraju matabolizam biljaka, pogotovo produkciju sekundarnih metabolita. Kao odgvor na stres izazvan promjenama u spoljašnjoj sredini biljke su razvile različite zaštitne mehanizme, a jedan od glavnih je produkcija sekundarnih metabolita. U kontrolisanim *in vitro* uslovima moguće je manipulisati rastom i razvićem biljaka, što najčešće rezultira povećanjem ili smanjenjem proizvodnje sekundarnih metabolita. U provodenom istraživanju, uspostavili smo brzi protokol za isklijavanje i uzgoj izdanaka vrste *Brassica oleracea* L. var. *italica* Plank. u *in vitro* uslovima. Tri, deset, dvadeset i trideset dana stari izdanci, kultivirani na tri različite Murashige-Skoog (MS) podloge te dvije vrste kalusa su korišteni za ekstrakciju. Za određivanje antioksidativnog potencijala petnaest etanolnih ekstrakata korištena je 2,2 '-difenil-1-pikrilhidrazil (DPPH$^{\bullet}$) radikalaska metoda. Ekstrakti tri dana starih izdanaka su imali najveći antioksidativni potencijal. S druge strane, ekstrakti izdanaka kultiviranih na osnovnoj MS podlozi su imali prooksidativnu aktivnost, koja bi se mogla pripisati prooksidativnim svojstvima nekih komponenti u prisustvu slobodnih iona prelaznih metala, tipa korištenog supstrata ali i biološkim uslovima u kojima dolazi do inteakcija.