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Hinokitiol and activated charcoal influence the microtuberization and growth of potato (*solanum tuberasum* cv. Agria) plantlets *in vitro*

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Abstract

The aim of this study was to assess the potential *in vitro* effects of activated charcoal and hinokitiol on microtuberization and growth of potato (*Solanum tuberasum* cv. Agria). Effect of these compounds was compared with abscisic acid, thidiazuron and paclobutrazol. The experiments were carried out in dark and light conditions. The number of microtubers induced per plantlet, microtuber dry and fresh weight, microtuber diameter and shoot fresh and dry weight were recorded at 60 days after culture initiation. Results under dark condition indicated that microtuberization occurred in all treatments except 0.1 percent of activated charcoal. On the other hand, activated charcoal significantly increased microtuber size at higher levels. Conversely, at lower concentration activated charcoal enhanced shoot growth. Hinokitiol at higher concentration (27 mg/l) increased only the size of the microtubers. However, the most microtuber numbers produced on media containing 2 µm of thidiazuron. However, these microtubers were smaller than microtubers obtained from media containing activated charcoal. Activated charcoal at (0.1, 0.5 and 1 percent), paclobutrazol (4 µM) and abscisic acid (1 µM) induced microtuber formation under light condition. Orthogonal comparison indicated that dark and light conditions affected all of measured traits differently. The best condition for microtuberization and shoot growth was provided in light and then dark condition.

Keywords: Microtuber, plant growth regulator, ACC-synthase, ACC-oxidase.
Abbreviations: ABA- abscisic acid, AC- activated charcoal, HIN- Hinokitiol, TDZ- Thidiazuron, and PBZ-Paclobutrazol.

Introduction

Potato microtuberization is a complex process, which is controlled by several factors including photoperiod (Seabrook et al., 1995), sucrose (Wang and Hu, 1982) nitrogen content of cultural media (Zarrabita et al., 1997), low temperature (Marious and Bodiaender, 1975) and gaseous condition of container (Paterson, 1970). Also, potato tuberization consists of several stages, which are under hormonal control (Levy et al., 1993). The role of some plant growth regulators in potato tuberization has previously been investigated. There are reports describing the importance of gibberlins, cytokinin, jasmonic acid and abscisic acid in tuber induction. Charcoal is a form of carbon which characterized by a high adsorptive capacity for gases, vapors and colloidal solids. Use of the charcoal can induce either positive or negative effects on cultures (Pan and Van Staden, 1998). In general, use of activated charcoal in tissue culture could be dealt with providing a dark environment (Nissen and Sutter, 1990), adsorption of undesirable or inhibitory substance (Tisserat, 1979), adsorption of plant growth regulator (Pan and Van Staden, 1998), releasing macro and micro- elements and inhibition of sucrose hydrolysis (Weatherhead et al., 1978).

Hinokitiol (β-tujaplicin) is a tropolon-related compound purified from the wood of *Chamaecyparis obtuse* and *Thja plicata*. Hinokitiol has been used as antibacterial agent in foods and cosmetics due to low toxicity in animals (Arimal et al., 2003). The inhibitory effects of hinokitiol on some plant growth stages such as seed germination have been reported (Sakagami et al., 2000). Moreover, hionkitiol can block the biosynthetic pathway of enzymes such as ACC-synthase and ACC-oxidase (Zobayd et al., 2001).

Thidiazouron (TDZ) is a synthetic herbicide with some growth regulatory functions in plants. Fundamental evidences about roles of TDZ in callus production (Capelle et al., 1983), tuberization (Kefi et al., 2000), embryogenesis (Gill and Saxena, 1993), shoot regeneration (Hamidoghli et al., 2011), bud break induction and adventitious bud formation (Murthy et al., 1998) are available.

Abscisic acid (ABA) is a natural plant hormone. This hormone can influence callus growth (Li et al., 1970), adventitious root and shoot formation, embryogenesis (Ammirato, 1988), but the conflicting effects of this compounds on potato tuberization has been reported (Kuda and Okazava, 1983).

Paclobutrazol (PBZ) is a triazole plant growth retardant compound (Fletcher et al., 2000). Different effects of PBZ on root (Pinhero et al., 1997), shoot (Davis et al., 1988) and leaf growth (Sebastian et al., 2002), photo assimilate allocation (Pinhero et al., 1997) and *in vitro* tuberization (Tekalign and Hammes, 2005), have been proven. The regulatory effects of activated charcoal and hinokitiol on potato tuberization have not been reported yet. Therefore, this investigation was undertaken to compare the effects of hinokitiol and activated charcoal with those of abscisic acid, thidiazuron and paclobutrazol on potato microtuberization.

Results

Microtuber numbers in dark condition

Media supplemented with 2 µM of TDZ significantly increased number of microtubers per plantlets. Activated charcoal at lower concentration inhibited the microtuber formation. ABA, PBZ and HIN did not significantly influenced microtuber formation. However, hinokitiol at 9 mg/l produced more microtubers than control.

Microtuber numbers in light condition

In general, fewer tubers induced under long photoperiod conditions. However, activated charcoal (0.1, 0.5 and 1 percent), ABA (at 1 µmol) and PBZ (at 4 µmol) significantly stimulated microtuber formation. Other treatments and control did not produce any microtuber under long photoperiod condition (Fig. 1).

Microtuber dry and fresh weight in dark condition

As shown in Fig 2 and 3, media supplemented with activated charcoal produced the largest microtubers. Maximum dry and fresh weight of microtubers obtained from plantlets grown on medium supplemented with 0.1 and 0.5 percent of activated charcoal. Also, hinokitiol at higher concentration (27 mg/l) significantly increased microtuber fresh weight. TDZ, PBZ and ABA did not influence fresh and dry weight of microtubers (Fig. 7).

Microtuber dry and fresh weight in light condition

Activated charcoal not only increased microtuber numbers, but also microtuber size. The largest microtubers achieved from plantlets grown on media containing 0.5 percent of activated charcoal (Fig. 2 and 3).

Microtuber diameter in dark condition

Application of activated charcoal at 0.5 percent and hinokitiol at 27 mg/l significantly increased potato microtuber size in dark condition. The largest (8.38 mm) and the smallest (1.6 mm) microtubers produced on plantlets grown on media containing 0.5 percent of AC and control, respectively. Other treatments had no significant effects on microtuber size (Fig. 4).

Microtuber diameter in light condition

As shown in Fig. 4 activated charcoal at 0.5 and 0.1 percent produced the largest microtubers with 8.2 and 6.6 mm of the diameter, respectively.

Shoot fresh and dry weight in dark condition

Media supplemented with PBZ (2 and 4 µM), ABA (4 µM) and hinokitiol (1, 3 mg/l) significantly reduced shoot growth. The highest shoot fresh and dry weight of plantlets obtained from media containing 0.1 percent of AC. In general, most of the treatments showed inhibitory effects on shoot growth.

Shoot fresh and dry weight in light condition

Unlike microtuberization, shoot growth was enhanced under long photoperiod condition. The maximum dry and fresh weight of shoot devoted to plantlets grown on media

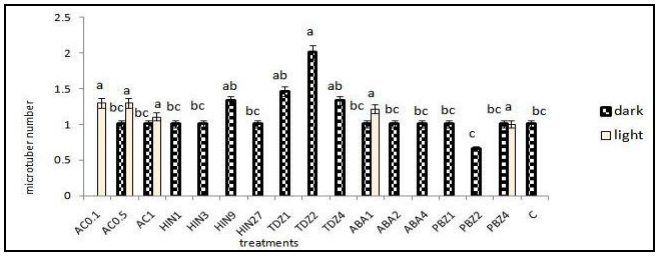

Fig 1. Effect of treatments on microtuber numbers.

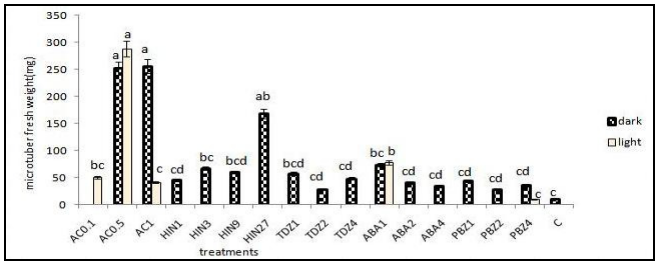

Fig 2. Effect of treatments on microtuber fresh weight.

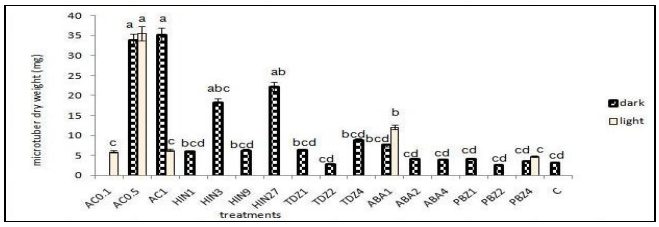

Fig 3. Effect of treatments on microtuber dry weight.

supplemented with 0.5 percent of AC. Hinokitiol at lower concentration (1 mg/l) increased shoot growth, but at higher concentrations (3, 9, 27 mg/l) did not influence shoot growth. TDZ (at 2 and 4 µM) and ABA (at 1 and 4 µM) significantly increased shoot fresh weight, but not shoot dry weight (Fig. 5 and 6).

Discussion

Activated charcoal under long photoperiod condition induced microtuberization of potato plantlets. The positive effect of AC on microtuberization might be due to adsorption of ethylene (a strong inhibitor for tuberization) and reduction of light in root medium. Reduction of light at the base of a shoot can provide a conducive environment to the accumulation of photosensitive auxin or co-factors (Pan and Van Staden, 1998). The lower proportion of AC arrested micro-tuberization of potato under *in vitro* condition. The non-selective effect of activated charcoal may result some negative effects on cultured explants and adsorption of tuber inductive compounds which can be responsible of these undesirable effects. Also, results showed that AC had a major role in increasing microtuber size under both light and dark conditions. The maximum fresh and dry weight of microtubers obtained at 0.5 and 1 percents of AC. AC at 0.5

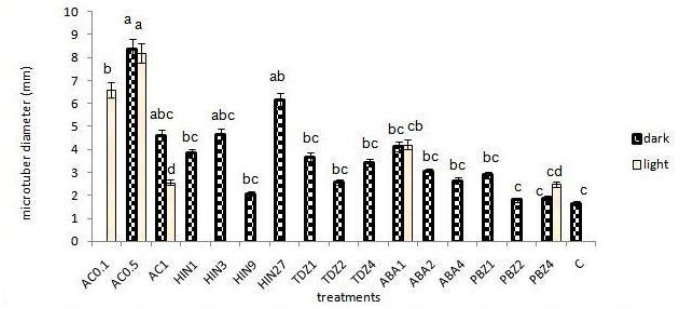

Fig 4. Effect of treatments on microtuber diameter (mm) under light and dark conditions.

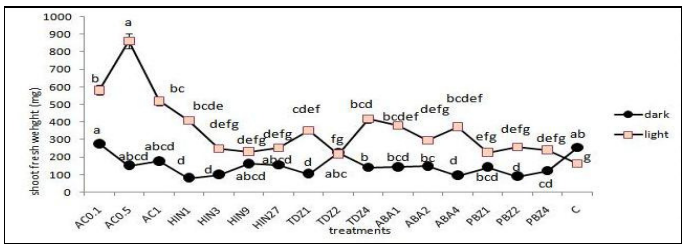

Fig 5. Effect of treatments on shoot fresh weight.

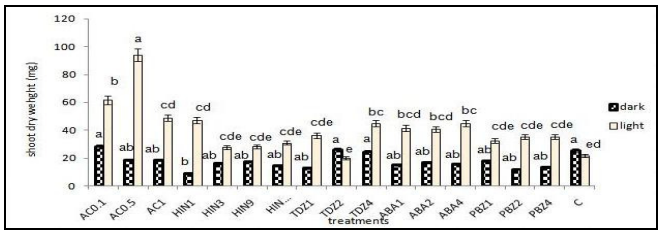

Fig 6. Effect of treatments on shoot dry weight.

percent showed similar result in light condition. The positive effect of activated charcoal on microtuber growth can be attributed to exclusion of light from root medium and inhibition of auxin degradation by light and consequent improvement of rooting ability of explants (Nissen and Sutter, 1990). Moreover, adsorption of undesirable substance such as ethylene released from explants or culture media by AC can enhance microtuber growth (Johansson et al., 1982). Similarly, activated charcoal enhanced shoot growth in both light and dark conditions. Some properties of this compound includes inhibition of sucrose hydrolysis during medium autoclaving and its ability to adsorption of phenolic compounds released from explants (Pan and Van Staden, 1998), which maybe contribute the promotion of shoot growth. Hinokitiol at higher level increased size of potato microtubers. The promoting effects of higher concentrations of hinokitiol on microtuber size probably may be the result of inhibitory effects of this compound on two key enzymes in ethylene biosyntheses, ACC-synthase and ACC-oxidase enzymes (Rabbany and Mizutani, 1998). Reduction of ethylene synthesis can provide a suitable condition for plantlets growth and development. The maximum numbers of microtuber achieved on media containing 2 µM of TDZ. Previously, the cytokinin-like activity of TDZ has been reported. Addition of cytokinin to tuberization media has also been reported to increase speed of tuber induction. Cell division is one of the early causing events of tuber initiation, The promoting effects of cytokinins in cell proliferation is likely involved in the early phase of tuber growth. Besides, cytokinins have been reported to have a role in sink creation by regulating the expression of genes implicated in assimilate partitioning. Therefore, these observations suggest that cytokinins may play a role in tuber formation by carbon fluxes towards the stolon cells (Davies, 2007). ABA and PBZ did not induce microtuber formation under dark condition, but increased microtuber formation under light condition. It has previously been reported that ABA-deficient mutants of potato are capable of tuberization under normal-hormone free condition, which indicates the presence of ABA is not necessary for tuber formation in dark condition (Davies, 2007), while under light condition ABA suppress GA_3 synthesis and may stimulates microtuberization. Also, the promotive effect of PBZ on tuberization of potato under light condition may be due to the antagonistic effects to gibberlins (Sebastian, et al., 2002). Reduction in stolon GA levels produce a longitudinal orientation of cell microtubules and microfibrils, thus triggering tuber formation by inducing cell expansion and cell division of the cells at the sub-apical region of the stolones (Davies, 2007). ABA, PBZ and hinokitiol decreased shoot growth under dark condition. The phyto-inhibitory effect of these compounds has been reported by several authors (Steffens et al., 1992., Sakagami et al., 2000., Li et al., 1970).

Material and methods

Plant material

Seed tubers of potato cv. Agria were planted in pots containing perlite, sand and soil in proportion of 1:1:2 in Research Greenhouse of Horticulture Department of Mohaghegh Ardabili University. Traditional cultural practices were done, when plant growth stages preceded, nodal segments were removed and cultured on MS medium for *in vitro* sterile plantlet production.

Explants and media sterilization

Explants were surface sterilized by immersion in 70 % (V/V) ethanol for 30 sec followed by immersion in 2% sodium hypochlorite solution for 20 min, and then rinsed three times with sterilized distilled water. Culture media were autoclaved at 121^{o}C for 20 min.

In vitro sterile plantlet production

Single-node cuttings, derived from plants grown in greenhouse, were cultured in test tubes containing hormone-free MS basal medium supplemented with 3% sucrose and 8 g/l agar. The cultures were maintained in a growth chamber at 22± 2 ^{o}C, 70-80 % relative humidity and 16:8 photoperiod under cool white fluorescent light (32 μmol m^{-2} s^{-1}).

Microtuberization

Single-node cuttings derived from *in vitro* sterile plantlets were cultured on MS basal medium supplemented with 6% sucrose, 8 g/l agar and various concentrations of hinokitiol (1, 3, 9 and 27 mg/l), Activated charcoal (0.1, 0.5 and 1 percent) and TDZ, PBZ and ABA (1, 2 and 4 µM). Cultures were incubated in growth chamber under two different light condition, half of the nodal segments were kept under

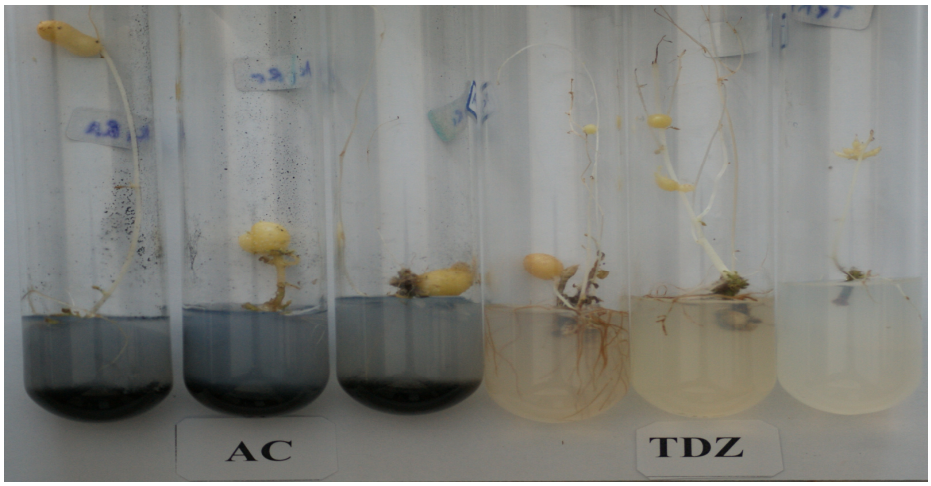

Fig 7. Effects of activated charcoal and thidiazuron on microtuber numbers and size under light condition.

16- days (long photoperiod) and half under completely dark condition. Temperature was regulated at 20±2°C.

Data recording

Microtuber numbers and diameter as well as shoot and microtuber fresh and dry weight were recorded at 60 days after culture initiation. Samples were dried at 70 °C for about 48 h to determine dry weight of microtubers and shoots.

Statistical analysis

Two separated experiments (light and dark) were established in a completely randomized design with 17 treatments and four replications. The means for treatment combinations was compared using the Waller-Duncan multiple range test (P>0.05). Data was subjected for analysis of variance and compare means using the statistical analysis system (SAS) program version 9.1 (SAS, 2003).

Conclusions

Addition of activated charcoal and hinokitiol in culture medium influenced shoot growth and tuberization of potato plantlets under *in vitro* condition. Investigation of mechanisms of absorption of AC and identification of the substance absorbed and released by activated charcoal will help our understanding on how charcoal acts in plants growth and development.

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