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Effect of activated charcoal and ascorbic acid on *in vitro* morphogenesis and o-dihydroxyphenols content in *Paphiopedilum insigne*

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Abstract: *Phenolic compounds* limit micropropagation of many orchids *in vitro*. The aim of the study was to estimate the effect of activated charcoal (AC); 1, 2 or 4 g/L) or ascorbic acid (AA; 10, 20 or 30 mg/L) added to the half strength MS medium on the growth and o-dihydroxyphenols content in *Paphiopedilum insigne in vitro*. A positive effect of AC on the shoot and root formation has been found. The highest multiplication rate (5.6 shoots/explant) and rooting frequency were obtained on medium containing 2 g/L of AC. However, AC reduced the leaf number as compared to the control. The lowest content of o-dihydroxyphenols was marked in *Paphiopedilum insigne* leaves when the shoots were grown on medium with 10 mg/L AA, followed by AC at 1 or 2 g/L.

Keywords: lady slipper; orchid; micropropagation; phenolic compounds; tissue culture

Paphiopedilum insigne, (Orchideaceae) is a pot plant with unique flowers shaped like slippers. It is also used as a cut flower (Ng, Saleh 2011).

In vitro techniques increase the availability of plants, but there are many difficulties like tissue or media browning due to phenolic compounds (Ndakidemi et al. 2014), what may lead to poor regeneration (Skrzypek et al. 2007).

To limit phenolic production, compounds like activated charcoal (AC) or ascorbic acid (AA) are used. Nongdam and Chongtham (2011) report that AC adsorb phenols. Its' positive effect was noted in *Phalaenopsis cornu-cervi* (Rittirat et al. 2012), *Cymbidium aloifolium* (Nongdam, Chongtham 2011).

Ascorbic acid catches free radicals from cutting tissues or phenols oxidation (Wojcieszyńska, Wilczek 2006). Its' positive influence was noted in *Brachylaena huillensis* (Ndakidemi et al. 2014) and *Tylophora indica* (Faisal et al. 2007).

The aim of the study was to estimate the effect of AC and AA on regeneration and phenolic compounds content in *Paphiopedilum insigne* cultures.

The explants were rosettes, 7–10 mm high and 12–15 mm wide with 3 leaves and 1–2 roots, taken from stable cultures started from asymbiotic seeds germination and passaged 3 times on regulators free 1/2 Murashige and Skoog (MS) medium (Murashige, Skoog 1962). Explants were placed on MS medium with macronutrients reduced by 1/2, with 0.05 mg/L thiamine, 0.25 mg/L pyridoxine, 0.25 mg/L nicotinic acid, 1 mg/L glycine, 50 mg/L myo-inositol, 15 g/L sucrose, 5 mg/L kinetin (KIN), and 1 mg/L benzylad-

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Table 1. The influence of ascorbic acid (AA) and activated charcoal (AC) on the number of regenerated plants and multiplication rate of *Paphiopedilum insigne*

Antioxidants	Mean No. of regenerated plants/treatment	Multiplication rate
Control	7.83[a*]	1.19[b]
AA 10 (mg/L)	7.83[a]	1.87[b]
AA 20 (mg/L)	8.33[a]	1.49[b]
AA 30 (mg/L)	8.67[a]	1.27[b]
AC 1 (g/L)	8.33[a]	1.98[b]
AC 2 (g/L)	9.50[a]	5.60[a]
AC 4 (g/L)	9.33[a]	2.60[b]

*Means followed by the same letter do not differ significantly at $P = 0.05$

enine (BA). Media were supplemented with AC (1, 2 or 4 g/L) or AA (10, 20 or 30 mg/L). The medium without additions was the control. The pH was adjusted to 5.7. Media were gelled with 6.75 g/L agar. There were 6 flasks with 10 plants per treatment, in two repetitions. The study lasted 16 weeks. A content of o-dihydroxyphenols in *P. insigne* leaves was done with Folin-Ciocalteu reagent according to Singelton and Rossi method (1965). Briefly, the 0.5 mL of the diluted sample was reacted with 2 ml of Folin-Ciocalteu reagent and 10 ml water for 3 min, and then 10 ml 10% of saturated sodium carbonate solution. The readings were done after 30 min at 760 nm with the use of Cary 100 Varian spectrophotometer. Caffeic acid was used as a reference standard, and the results were expressed as caffeic acid equivalent.

Cultures were kept at 28ºC ± 2 ºC and 16-hour photoperiod with the light intensity of 30 μmol/m^2/s.

The data was analyzed with Statistica 13 (StatSoft), according to one – way ANOVA. The significance of differences was estimated with the Tukey's confidence intervals at the level of significance $\alpha = 0.05$.

Activated charcoal and ascorbic acid had no effect on the number of regenerated *P. insigne* plants, but AC significantly enhanced the shoot multiplication (Table 1). The highest shoot number (5.6) was noted in the presence of 2 g/L of AC. Similar results were previously obtained by Nongdam and Chongtham (2011) in *Cymbidium aloifolium* and by Fasal et al. (2007) in *Tylophora indica*. We observed that the shoot of *P. insigne* grown in the presence of AC produced less leaves as compared to control, but only AC at 1 g/L influenced formation of bigger leaves (Table 2). The positive effect of AC on rosettes height was noted in *Cattleya walkeriana* (de Faria et al. 2002) or *Miltonia flavescens* (Morales et al. 2003). Rittirat et al. (2012) reported that in presence of AC *Phalaenopsis cornu-cervi* leaves were longer and wider.

As was shown in Table 2, the addition of AC to the medium resulted in an increase of the rooting rate by 20%, whereas AA slighty reduced this process as compared to the control. Activated charcoal had also positive effect on the root length and weight.

The positive effect of AC on rooting *in vitro* was noted by many authors. Yan et al. (2006) proved that AC darkened media promoting roots development. A higher number of roots in presence of AC was observed in *Phalaenopsis cornu-cervi* (Rittirat et al. 2012), *Miltonia flavescens* and *Oncidium trulliferum* (Morales et al. 2003)

Table 2. Influence of ascorbic acid (AA) and activated charcoal (AC) on morphological features of *Paphiopedilum insigne* in tissue culture

Antioxidants	Leaves			Weight of rosettes (mg)	Roots			
	Number/ plant	Length (mm)	Width (mm)		Presence (%)	Number/ plant	Length (mm)	Weight (mg)
Control	3.76[a*]	12.04	5.03	80.03[b]	75[ab]	1.89[b]	3.61[c]	11.98[b]
AA 10 (mg/L)	2.31[b]	9.69	3.97	89.57[b]	74[ab]	2.26[b]	3.61[c]	19.05[b]
AA 20 (mg/L)	3.23[a]	10.78[b]	4.45	79.78[b]	72[b]	1.81[b]	3.94[c]	17.02[b]
AA 30 (mg/L)	3.75[a]	12.41	4.69	89.89[b]	77[ab]	2.45[b]	4.97[c]	27.21[b]
AC 1 (g/L)	2.55[b]	12.46	4.82	187.55[a]	90[ab]	4.27[a]	10.86[a]	153.12[a]
AC 2 (g/L)	2.35[b]	7.33	3.04	219.14[a]	95[a]	5.02[a]	8.86[b]	140.15[a]
AC 4 (g/L)	2.56[b]	11.44[b]	4.27	173.23[a]	95[a]	3.96[a]	8.48[b]	113.98[a]

*Means followed by the same letter do not differ significantly at $P = 0.05$

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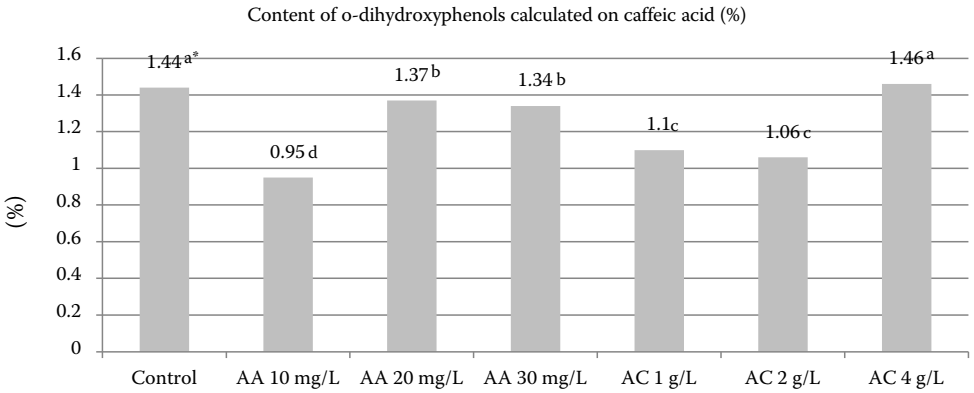

Figure 1. The influence of ascorbic acid and activated charcoal on o-dihydroxyphenols content in *Paphiopedilum insigne* leaves *in vitro*

*Means followed by the same letter do not differ significantly at $P = 0.05$

The biochemical analyses showed that the content of o-dihydroxyphnols in *P. insigne* leaves *in vitro* was the lowest in the presence of 10 mg/L AA (0.95%)followed by AC at 1 or 2 g/L AC (1.10 and 1.06% respectively).

Similar results were obtained in *Cymbidium* (da Silva 2013). The positive effect of 100 mg/L AA on the reduction of phenolic compounds production was observed by Ngomuo et al. (2014) in *Musa* spp. *in vitro*. Ndakidemi et al. (2014) confirmed that 200 mg/L of AA decreased phenols in *Brachylaena huillensis* and Ko et al. (2009) in *Cavendish banana* cv. *Formosana* cultures. AC has a perfect adsorption ability towards phenolic compounds, but it depends on interactions between the compounds or other effects (Dąbrowski et al. 2005). In the presented research AC at 1–2 g/L reduced the amount of o-dihydroxyphenols, while AC at 4 g/L had no effect on this process, as compared to the control. Different effects were noted by Abdelwahd et al. (2008) in *Vicia faba*, as AC 10 g/L significantly reduced browning of explants and had a positive effect on regeneration. da Silva (2013) reported that on 1 g/L AC media browning was not observed in *Cymbidium* tissue culture .

CONCLUSION

Activated charcoal and ascorbic acid affect micropropagation of *Paphiopedilum insigne*. Multiplication rate was the highest in the presence of 2 g/L AC, therefore such medium is advised for plants cultivation *in vitro*. The rosettes with the highest number of leaves were noted on the control media and in presence of 20 or 30 mg/L of AA, while AC at 1 g/L increased the number and length of roots.

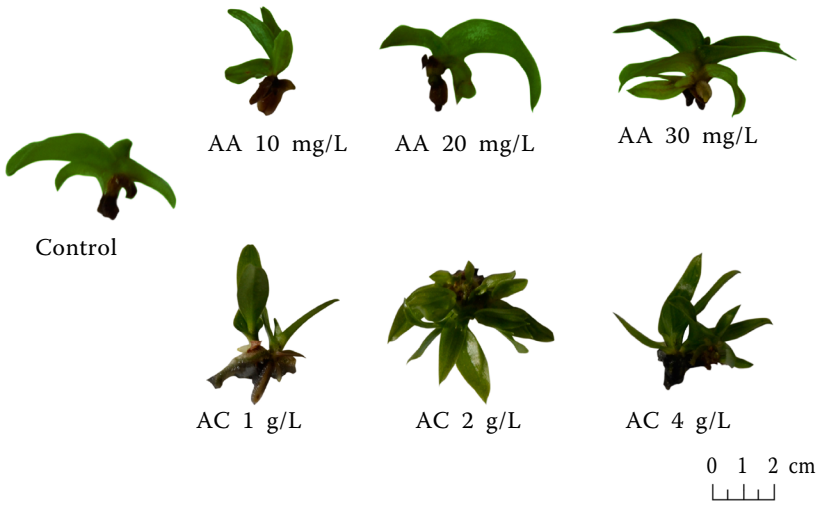

Figure 2. The influence of ascorbic acid and activated charcoal on morphological features of *Paphiopedilum insigne* in tissue culture

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The amount of o-dihydroxyphenols in leaves was the lowest on the media with 10 mg/L AA. The addition of antioxidants to the media, except the highest amount of AC, decreased the content of o-dihydroxyphenols in leaves.

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