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IN VITRO GROWTH OF *ALOE BARBADENSIS* MILL.: THE EFFECT OF ACTIVATED CHARCOAL ON MEDIUM PH, NITROGEN UPTAKE AND ELEMENTS CONTENT OF SHOOTS

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Abstract

Activated charcoal was added to the elongation medium of *Aloe barbadensis* micropropagated *in vitro*. Its inclusion significantly increased shoot growth. In order to explain the improvement of the growth performance, the effect of activated charcoal on medium pH and elements uptake during the culture period was investigated. It was found that activated charcoal prevented the drop of the medium pH which occurred in its absence, probably because it decreased the ammonium:nitrate (NH_4^+ : NO_3^-) uptake ratio. In fact, a significant negative correlation between NH_4^+ : NO_3^- uptake ratio and medium pH occurred. Moreover, activated charcoal significantly affected the N, K, Ca, Na, Mn, Fe, B, and Zn concentration in the shoots, while days of culture influenced N, K, P, Na, Fe, Zn, and Cu presence in *A. barbadensis* shoots.

Key words: ammonium to nitrate ratio, micropropagation, nutrients uptake

INTRODUCTION

Aloe barbadensis Mill. (Liliaceae) is an important ornamental and medicinal plant. This monocotyledonous species grows in rosette shape around a small portion of stem no greater than 5.0 cm (www.botany.com/aloe.htm). The leaves, usually 12.0 - 16.0 per plant, are simple, triangular, succulent, thick, with narrow lanceolate mucro tip, 30.0 - 60.0 cm long, and 5.0 - 12.0 cm wide at the base and 0.8 - 3.0 cm thick. The margins of the leaves have sharp triangular teeth about 2.0 mm long. The main root is 4.0 - 10.0 cm long and 4.0 - 5.0 cm in diameter, the rhizosphere is concentrated at a depth of 15.0 - 20.0 cm (Ahlawat and Khatkar 2011). Flowers are 2.5 - 3.0 cm long, yellow, grouped in clusters on a single erect stem about 1.0 m long. The fruit is a triangular capsule containing numerous seeds. *A. barbadensis* reproduces only by vegetative propagation as the seeds are not viable due to the sterility of the male flowers (Keijzer and Cresti 1987). Adventitious shoots are formed on the underground stem but their formation is limited and with a seasonal frequency for which this technique is very slowly and expensive (Hashem Abadi and Kaviani 2010). *In vitro* culture is an alternative propagation method, which facilitates its large scale production in limited time and space (Malda et al. 1999). Some researchers micropropagated *A. barbadensis* through axillary shoot formation (Natali et al. 1990, Thind et al. 2008, De Oliveira et al. 2009, Hashem Abadi and Kaviani 2010). It is generally accepted that the ultimate success of a micropropagation protocol depends on the satisfactory establishment of microplants *in vivo* (Ramirez-Malagon et al. 2001). Percentage survival is often used as a measure of this. De Oliveira et al. (2009) reported that it is dependent on shoot quality. Similarly, they reported that the acclimatization of aloe plants was better for larger ones.

Activated charcoal (AC) is largely used *in vitro* with the aim to improve growth, multiplication, elongation, and rooting of shoots before their establishment *in vivo* (Hemphill et al. 1998, Quoirin et al. 2001, Gubbuk and Pekmezci 2006). The effect of AC in the culture medium has been attributed to the establishment of a dark environment simulating soil conditions (Dumas and Monteuuis 1995, Yan et al. 2006), adsorption of inhibitory substances in the culture medium (Fridborg et al. 1978, Pan and van Staden 1998), regulation of plant growth regulators (PGRs) levels in the culture medium (Van Winkle and Pullman 2005), alteration of medium hydrogen ion concentration (pH) (Owen et al. 1991) and modification of nutrient adsorption (Eymar

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et al. 2000, Van Winkle et al. 2003, Van Winkle and Pullman 2003). According Hashem Abadi and Kaviani (2010) addition of AC in the culture medium of *A. barbadensis* improves height, fresh weight, and root number of micropropagated shoots and also ensures a better plant growth during acclimatization (Borgognone et al. 2010). Nevertheless, no data are available concerning the *in vitro* effects of AC on morphological and physiological response of *A. barbadensis* shoots in relation to the putative chemical changes occurring in the culture medium. It is contended that AC may prevent the initial acidic shift of the medium by regulating the availability of both ammonium and nitrate. To verify this hypothesis an *in vitro* experiment was carried out to compare the growth and the element composition of the shoots, the final pH, electrical conductivity (EC), NH_4^+, and NO_3^- concentration in the medium after both 20 and 40 days in culture in presence or absence of AC.

MATERIALS AND METHODS

Plant material and culture conditions

A. barbadensis shoots (2.0-3.0 cm long) were micropropagated on full-strength MS (Murashige and Skoog 1962) medium including vitamins to which sucrose (30.0 g l^{-1}), N^6-benzyladenine (BA, 2.0 mg l^{-1}), indole-3-acetic acid (IAA, 0.2 mg l^{-1}), and bacto agar [(7.0 g l^{-1}) (Sigma Chemical Co., MI, Italy)] were added. The pH was adjusted to 5.7 ± 0.1 with 0.1N HCl or 0.1N NaOH before autoclaving the medium at 121°C and 105 kPa for 20 min. After 45 days in the medium, new developed adventitious shoots (1.0-1.5 cm long) were excised and placed on one half-strength MS medium devoid of growth regulators but containing 30.0 g l^{-1} sucrose and 7.0 g l^{-1} agar to promote elongation and rooting. Four treatments were established as follows:

- no AC in the medium, culture period of 20 days;
- no AC in the medium, culture period of 40 days;
- 1% (w/v) AC (Duchefa, Haarlem, The Netherlands) in the medium, culture period of 20 days;
- 1% (w/v) AC in the medium, culture period of 40 days.

Eight shoots were placed in each 500.0 ml glass vessel containing 200 ml of medium and transferred to a growth chamber maintained at 24 ± 1°C under a 16 h photoperiod provided by cool-white fluorescent lamps emitting a photosynthetic photon flux density of 40 μmol m^{-2} s^{-1}. Five replicates per treatment were established. The pH was adjusted as described above after the addition of AC and prior to autoclaving for 20 min (at 121°C/105 kPa).

Measurements and analysis

Values of pH and EC of the medium were measured after sterilization using a pH meter (GLP21, Crison Instruments, Barcelona, Spain) and an EC meter (HI 86304, Hanna Instruments, Padova, Italy), respectively. After both the 20 and 40 day culture periods, shoot length, number of leaves and roots, root length, fresh and dry weight were determined for each implanted shoot. Dry weight of each shoot was determined after drying for 48 h at 70°C. Daily shoot growth was calculated according to the method of Brito et al. (2009) as the final shoot length minus the initial shoot length divided by the number of days of culture. Growth index (GI) was calculated as described by Russowski et al. (2006) as the final length minus the initial length divided by the initial length. The root : shoot ratio was obtained dividing the root length by the shoot length.

At the end of the elongation and rooting period the remaining medium in each vessel was collected to determine pH, EC, and the content of NO_3^- and NH_4^+. For pH and EC measurements, spent medium has been centrifuged before analysis. Nitrate and ammonium concentrations were analyzed in the liquid fraction of substrate which was obtained by the medium centrifugation for 20 min at 15000 × *g* and filtration through Whatman filter. Nitrate and ammonium concentrations in the liquid fraction of the medium were analyzed by spectrophotometry (Helios Beta, Spectrophotometer, Thermo Electron Corporation, England). Nitrate was assayed using the salicylic acid-sulfuric acid method (Cataldo et al. 1975). Ammonium was determined by the phenol-hypochlorite reaction (Weatherburn 1967). Nitrate and ammonium uptake by plantlets was calculated by measuring the difference between nutrient residual after 20 and 40 days of culture and nutrient supplied by MS medium and the uptake was expressed on the base of plant dry weight (Adelberg et al. 2010).

All dried shoots per replicate were ground in a Wiley mill to pass through a 20-mesh screen and stored to analyse the total nitrogen and elements content. The N concentration of dried plant tissues was determined after mineralization with sulphuric acid by the Kjeldhal method (Bremner 1965). Dried tissues (0.20 g) were analysed for the following macro- and microelements: K, P, Mg, Ca, Na, Mn, Fe, B, Zn, and Cu. Their concentration was determined by dry ashing at 400°C for 24 h, dissolving the ash in HNO_3 1 : 20 v/v and assaying the solution obtained by an inductively coupled plasma emission spectrophotometer (ICP Iris: Thermo Optek, Milan, Italy; Karla 1998). The mineral content was expressed in mg (macroelements) or μg (microelements) g^{-1} of dry weight.

Statistical analysis

All data were analyzed by using the SPSS software package (SPSS 16.0 for Windows) (Field 2005). Data were subjected to two-way analysis of variance (ANOVA). Percentage values of dry matter were arcsine-transformed before analysis of variance. Treatment means were compared when the *F*-test statistic

for treatment was significant, with least squares means separated using the least significant difference method ($p \leq 0.05$). Correlation analyses were conducted between NH_4^+: NO_3^- uptake and pH using the SigmaPlot 8.0 package (SigmaPlot, Richmond, CA, USA).

RESULTS

Growth parameters

After 20 days of culture, shoot length has almost doubled with AC compared to the treatments without AC and a similar trend was also found after 40 days *in vitro*. Shoots fresh weight significantly increased from 0.3 to 1.2 g in presence of AC after 20 days and from 0.6 to 2.2 g after 40 days of culture and the daily shoot growth was approximately 3 times higher with AC. Dry matter and root number were not influenced by AC treatment but significant differences were observed respect to the culture time (Table 1).

Growth index was significantly influenced by the interaction between AC and days of culture with the highest value after 40 days in the presence of AC (Fig. 1). After 40 days of culture without AC it was observed the same value of GI that after 20 days of culture with AC. The lowest GI value was recorded after 20 days of culture in the medium without AC. Root to shoot length ratio is presented in Fig. 2 with a significant interaction between the experimental factors. After 20 days of culture the value of the ratio was not different between the presence or absence of AC in the medium, while, after 40 days of culture, the ratio for the treatment with AC was 0.5 while without AC was 1.9, showing a significant difference induced by the use of AC (Fig. 2).

Medium pH, EC, and nitrogen uptake

Medium pH measured after autoclaving was 5.71 and 6.18, respectively with and without AC (data not shown), and it changed during shoots cultivation. The presence of AC in the medium had a significant buffer-

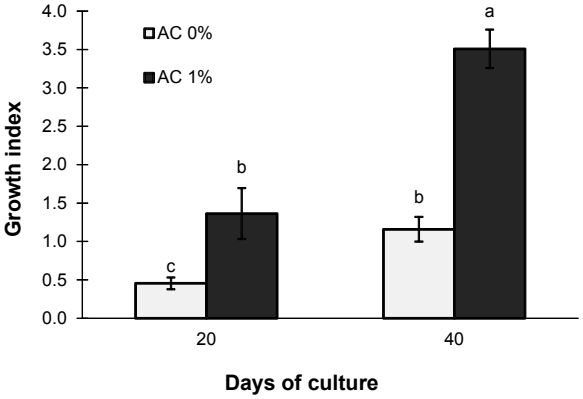

Fig. 1. Combined effects of activated charcoal (AC) and days of culture on growth index.
Means with common letter between columns are significantly different according to MSD test ($p \leq 0.05$). Values are means (± SE) of five replicates.

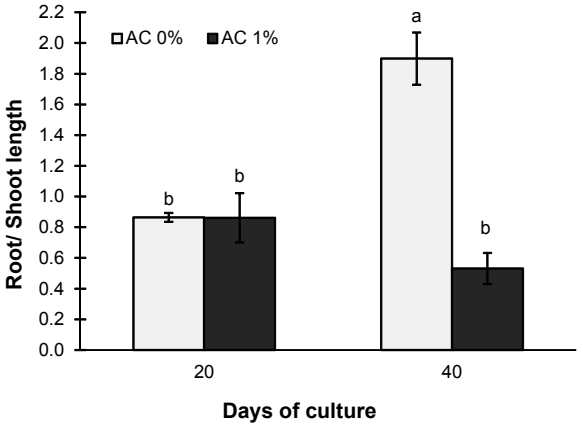

Fig. 2. Combined effects of activated charcoal and days of culture on root to shoot length ratio.
Means with common letter between columns are significantly different according to MSD test ($p \leq 0.05$). Values are means (± SE) of five replicates.

Table 1. Effects of activated charcoal and days of culture on biometrical traits of *Aloe barbadensis* shoots.

Activated charcoal (AC) (%)	Days of culture (D)	Shoot length (mm)	No. of leaves	Fresh weight (g shoot^{-1})	Dry matter (%)	Daily shoot growth (mm d^{-1})	No. of roots
0	20	18.2 ± 0.86	4.9 ± 0.25	0.3 ± 0.05	6.9 ± 0.71	0.33 ± 0.03	3.1 ± 0.22
0	40	29.9 ± 1.20	5.7 ± 0.29	0.6 ± 0.08	5.3 ± 0.69	0.12 ± 0.02	4.4 ± 0.16
1	20	33.5 ± 4.40	5.6 ± 0.29	1.2 ± 1.11	6.2 ± 1.00	0.25 ± 0.05	2.6 ± 0.77
1	40	57.1 ± 3.40	5.2 ± 0.39	2.2 ± 0.43	4.2 ± 0.58	0.05 ± 0.01	3.9 ± 0.46
Significance							
AC		***	ns	***	ns	***	ns
D		***	ns	*	*	ns	*
AC*D		ns	ns	ns	ns	ns	ns

ns, (*), (***) are non significant or significant at $p < 0.05$ or 0.001, respectively.
Values are means (± SE) of five replicates.

Table 2. Effects of activated charcoal and days of culture on pH and electrical conductivity (EC) of the culture medium, the uptake of nitrate and ammonium and the ratio between ammonium and nitrate uptake.

Activated charcoal (AC) (%)	Days of culture (D)	pH	EC (dS m^{-1})	NO_3^- uptake (mg g^{-1}dw)	NH_4^+ uptake (mg g^{-1}dw)	NH_4^+/NO_3^- uptake
0	20	4.55 ± 0.14	3.87 ± 0.39	16.32 ± 1.74	21.70 ± 1.47	1.34 ± 0.07
0	40	4.40 ± 0.07	2.97 ± 0.17	17.17 ± 1.07	18.53 ± 0.93	1.10 ± 0.13
1	20	5.53 ± 0.34	3.05 ± 0.24	23.70 ± 0.84	20.68 ± 0.85	0.88 ± 0.07
1	40	5.08 ± 0.17	1.68 ± 0.04	23.81 ± 0.94	16.94 ± 2.44	0.70 ± 0.08
Significance						
AC		**	**	**	ns	**
D		**	ns	ns	ns	*
AC*D		ns	ns	ns	ns	ns

ns, (*), (**), are non significant or significant at $p < 0.05$ or 0.01, respectively.
Values are means (± SE) of five replicates.

ing effect on pH (ranging from 5.53 after 20 days to 5.08 after 40 days of culture) while the pH values dropped to a value lower than 4.60 in the medium lacking AC (Table 2).

After autoclaving, the EC value was 5.20 dS m^{-1} in the medium with AC and 4.77 dS m^{-1} in the medium without AC (data not shown) and these values were affected by the presence of AC (Table 2).

The uptake of nitrate was higher for the shoots grown on the medium containing AC with respect to the control without AC while the ammonium uptake was not affected by the treatments (Table 2).

Both AC treatment and the length of the culture had a significant effect on ammonium to nitrate uptake ratio. In fact, this ratio was significantly lower for the treatment with AC (0.88 and 0.70 after 20 and 40 days of culture, respectively) respect to the control without AC (1.34 and 1.10 after 20 and 40 days of culture, respectively).

After 20 days of culture, there was a significant linear relationship between ammonium to nitrate uptake ratio and medium pH; the increase of the NH_4^+: NO_3^- ammonium to nitrate uptake ratio corresponded to a reduction of the medium pH (Fig. 3).

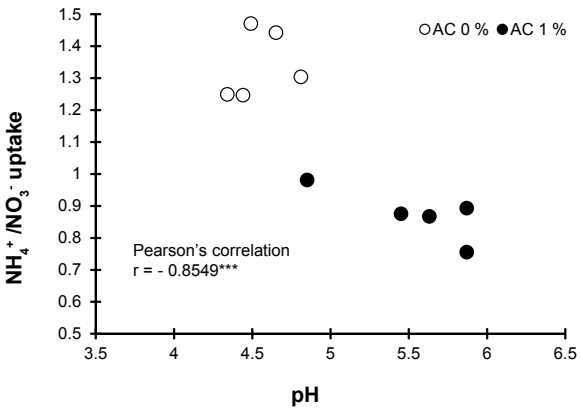

Fig. 3. Correlation analysis between ammonium to nitrate uptake ratio and medium pH after 20 days of culture in presence or absence of activated charcoal in the elongation and rooting medium of *Aloe barbadensis* shoots.
*** indicates the Pearson's correlation coefficient is statistically significant at $p < 0.001$.

Mineral nutrient content

Total N content of *A. barbadensis* shoot was affected by AC and days of culture (Table 3): plants grown in presence of AC accumulated about 34 mg g^{-1} dw of N, similarly to what accumulated after 40 days of culture without AC (32.1 mg g^{-1} dw). K content was higher in shoots cultured in absence of AC after 20 days of culture and it descreased with AC (Table 3). P content was affected by the interaction between AC treatment and the days of culture; the highest value of P concentration was detected at 20 days of culture in absence of AC (9.3 mg g^{-1} dw) while the lowest one was recorded after 40 days of culture in absence of AC (5.7 mg g^{-1} dw). In presence of AC, P concentration did not change over the culture period (7.1 and 7.0 mg g l^{-1} dw after 20 and 40 days of culture, respectively). Also, Mg concentration was affected by the interaction between the two experimental factors. In fact, Mg concentration decreased after 40 days of culture on medium lacking in AC passing from 2.1 mg g^{-1} dw at 20 days to 1.7 mg g^{-1} dw at 40 days. Mg concentration did not change during the culture period in presence of AC (1.8 mg g^{-1} dw at 20 days and 1.9 mg g^{-1} dw at 40 days). Ca content was significantly higher in shoots cultured on medium without AC (7.7 mg g^{-1} dw after 20 days and 6.6 mg g^{-1} dw after 40 days of culture) (Table 3). Na concentration in aloe tissues was affected by treatments (Table 3) and by the interaction between AC treatment and days of culture; in presence of AC, the Na content was 1.1 mg g^{-1} dw after 20 days of culture

Table 3. Effects of activated charcoal and days of culture on the final concentration of macroelements of *Aloe barbadensis* tissue.

Activated Charcoal (AC) (%)	Days of culture (D)	Macroelements (mg g^{-1} dw)					
		N	K	P	Mg	Ca	Na
0	20	24.8 ± 3.12	42.0 ± 2.42	9.3 ± 0.35 a	2.1 ± 0.13 a	7.7 ± 0.70	1.5 ± 0.12 a
0	40	32.1 ± 1.32	34.3 ± 1.42	5.7 ± 1.15 b	1.7 ± 0.06 b	6.6 ± 0.34	1.5 ± 0.04 a
1	20	33.4 ± 1.15	33.6 ± 1.10	7.1 ± 0.31 b	1.8 ± 0.10 b	5.4 ± 0.28	1.1 ± 0.05 b
1	40	35.4 ± 1.12	31.6 ± 0.86	7.0 ± 0.47 b	1.9 ± 0.06 ab	5.5 ± 0.06	1.5 ± 0.04 a
Significance							
AC		*	**	ns	ns	**	*
D		*	*	*	ns	ns	*
AC*D		ns	ns	*	*	ns	*

ns, (*), (**) are non significant or significant at $p < 0.05$ or 0.01, respectively.
Means with a common letter within columns are not significantly different according to MSD test ($p = 0.05$).
Values are means (± SE) of five replicates.

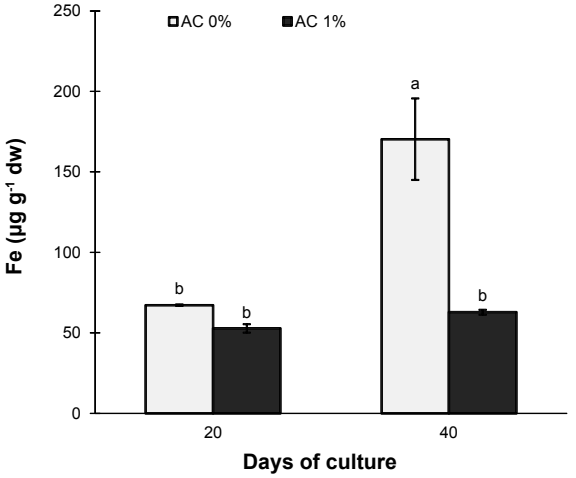

Fig. 4. Combined effects of activated charcoal and days of culture on Fe concentration in *Aloe barbadensis* shoots. Means with common letter between columns are significantly different according to MSD test ($p \leq 0.05$). Values are means of five replicates.

and it increased to 1.5 mg g^{-1} dw after 40 days, while without AC, the Na concentration was 1.5 mg g^{-1} dw for both times of culture.

Mn and B were significantly higher in tissues not treated with AC (Table 4). Zn concentration was higher without AC and generally for a culture period of 40 days. Cu concentration in the tissues was affected only by culture period while Fe concentration was significantly affected by AC and days of culture (Table 4) and also the interaction between the two experimental factors was significant (Fig. 4). A very high increase of Fe concentration was detected at the end of 40 days of culture only in combination with the absence of AC in the medium (170.3 µg g^{-1} dw) (Table 4).

DISCUSSION

AC is commonly employed in plant tissue culture to improve proliferation, growth, and rooting of explants (Thomas 2008). AC is produced by destructive distilla-

Table 4. Effects of activated charcoal and days of culture on the final concentration of microelements of *Aloe barbadensis* tissue.

Activated Charcoal (AC) (%)	Days of culture (D)	Microelements (µg g^{-1} dw)				
		Mn	Fe	B	Zn	Cu
0	20	174.5 ± 21.75	67.2 ± 0.43 b	29.3 ± 1.07	66.6 ± 3.94	3.3 ± 0.25
0	40	161.1 ± 20.78	170.3 ± 25.30 a	35.8 ± 5.63	84.5 ± 7.51	7.6 ± 1.34
1	20	113.2 ± 13.27	52.8 ± 2.67 b	21.7 ± 1.47	43.6 ± 1.15	4.3 ± 0.34
1	40	133.7 ± 7.14	62.8 ± 1.49 b	23.7 ± 0.66	59.7 ± 1.56	6.6 ± 1.27
Significance						
AC		*	***	*	***	ns
D		ns	**	ns	**	**
AC*D		ns	**	ns	ns	ns

ns, (*), (**), (***) are non significant or significant at $p < 0.05$, 0.01 or 0.001, respectively.
Means with a common letter within columns are not significantly different according to MSD test ($p = 0.05$).
Values are means (± SE) of five replicates.

tion of woods, peat, lignite, nut shells, bones, vegetables or other carbonaceous matter and then activated by the removal of impurities and the oxidation of carbon surface. The result is a AC with highly developed porous structure and large specific area and with a considerable adsorptive power (Pan and van Staden 1998).

An improved growth performance of *A. barbadensis* shoots was obtained by adding AC in the culture medium. Previously, AC had been used *in vitro* to enhance the percentage of shoots elongation of *Acacia mearnsii* (Quoirin et al. 2001) and the adventitious shoots length in banana (Gubbuk and Pekmezci 2006). Hashem Abadi and Kaviani (2010) compared the effect of different phenolic attractive substances on the length of *Aloe vera* plantlets and the best result was achieved by including 0.2 % (w/v) AC in the shoot proliferation medium. The addition of AC to the culture medium had also promoted the growth and the increase of the fresh weight in rhizomes of *Cymbidium forrestii* (Paek and Yeung 1991), in *Anoectochilus formosanus* shoots (Ket et al. 2004), and in microtubers of *Dioscorea nipponica* (Chen et al. 2007).

Absence of AC led to higher elongation of the roots rather than the shoots. Root to shoot ratio is an indicator of biomass allocation in plants (Nuruddin and Chang 1999) and, usually, plants under environmental stress easily accumulate more biomass in the roots as reported for herbaceous mimosa (Nuruddin and Chang 1999). Probably AC had a role in setting optimal conditions for *in vitro* elongation and rooting and in reducing stress factors of the *in vitro* environment (Hazarika 2006, Krishna et al. 2008) which may lead to growth inhibition and physiological disorders. In fact even pH changes, usually drifting to an acidic range following the culture period, can affect the development of plants (Owen et al. 1991, Shibli et al. 1999) and the results showed the effect of AC on the stabilization of medium pH to an optimal level (5.0-5.5). This buffering ability is mainly related to the adsorptive capacity of the AC porous structure towards cations and substances released during autoclaving or during the culture period by the explants. The capture of cations may affect the nutrient balance in the medium (Van Winkle et al. 2003, Van Winkle and Pullman 2003) and the uptake of the two nitrogen sources (NH_4^+ and NO_3^- ions) provided by the MS medium (Eymar et al. 2000). In fact the ratio between the shoot uptake of NH_4^+ and NO_3^- was significantly lower in the treatment with AC and the pH of the medium did not decrease during the experiment while in absence of AC, the NH_4^+ to NO_3^- uptake ratio was higher and the medium pH dropped as shown by the negative correlation in Fig. 3. Experiments of nitrogen nutrition with *Picea abies* seedlings led us to hypothesize that the presence of NH_4^+ and the low pH of the nutrient solution decreased the assimilation of NO_3^- (Peuke and Tischner 1991). In our experiment, *A. barbadensis* shoots were exposed to similar condition in absence of AC: low pH of the medium and putative higher availability of NH_4^+ ions. The hypothesis suggested is that *A. barbadensis* shoots request a lower NH_4^+: NO_3^- ratio than that provided through MS medium to better assimilate nitrogen and therefore rapidly grow.

The nitrate uptake by shoots (Table 2) and the total nitrogen content (Table 3) of *A. barbadensis* tissues were heightened by the effect of AC. Even in *Camellia sinensis* plants Ruan et al. (2007) found a close relation between greatest growth rate, high concentration of total nitrogen in tissues and a good absorption rate of NO_3^-.

However, despite the significant buffer effect evidenced by the experiments, the involvement of other factors can not be excluded to explain the promoting role of AC in *in vitro* shoots growth. Previous investigations concerning AC focused also on the effects of the darkening of the root environment (Dumas and Monteuuis 1995, Yan et al. 2006), the removal of growth inhibitors and hormonal excess from the medium (Fridborg et al. 1978) and the hydrolysis of sucrose to glucose and fructose upon autoclaving (Wann et al. 1997, Pan and van Staden 1999).

The differences observed in the mineral composition of shoots may be partially due to the AC influence; this can significantly impact available element composition through adsorption, pH alteration and contribution of impurities (Van Winkle and Pullmann 2003). The ability of AC to capture cations on its particle surface may have reduced the uptake by the explants and therefore result in a lower concentration of cations in tissues. Moreover, in absence of AC, the higher concentrations of iron and boron in shoots may be correlated with a major uptake rate of these ions when the pH of the medium drops. The large increase of iron in the tissues after 40 days of culture on medium lacking AC (Fig. 4) may be a further factor of stress responsible for the growth inhibition. Iron in tissues catalyzes the generation of active oxygen species via the Fenton Reaction leading to oxidative stress and growth reduction (Wu et al. 1998).

The growth stimulation obtained by the addition of AC in the elongation and rooting medium of *A. barbadensis* shoots is probably due to a synergistic effect of several factors: (i) buffer ability; (ii) influence on nitrogen utilization; (iii) limitation of stress conditions of *in vitro* culture.

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