***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. This may be attributed to several factors including alteration of medium pH and modification of nutrient availability. It was found that activated charcoal prevented a drop medium pH which occurred observed in its absence, probably because it decreased the ammonium:nitrate (NH4+:NO3-) uptake ratio. In fact, a significant negative correlation between NH4+/NO3- uptake ratio and medium pH was occurred. Moreover, activated charcoal significantly affected the elements’ concentration in the shoots, given its ability to capture cations on its particle surface and therefore to reduce their concentration in tissues. In the absence of activated charcoal Fe accumulation in the shoots was detected after 40 days of culture.

**Keywords**

Aloe vera; Micropropagation; Nutrients uptake; Ammonium to nitrate ratio

**Running title** Activated charcoal on *in vitro* growth of *Aloe barbadensis*

**Introduction**

*Aloe barbadensis* Mill. is an important medicinal plant belonging to the Liliaceae family. It has been used for centuries in traditional and folk medicine to treat several health disorders.(Ref) Nowadays its leaves are processed by industry as a source of pharmaceutical, cosmetics and healthy food products. Aloe is commonly propagated through lateral buds but this technique doesn’t satisfy the industry’s biomass demand. (Ref) *In vitro* culture is an alternative propagation method which facilitates its large scale production in limited time and space (Ref).

Some researchers micropropagated *A. barbadensis* with variable results; a phenomenon ascribed to the type and concentration of plant growth regulators added to the culture medium and the number of subcultures undertaken (Meyer and van Staden 1991; Chaudhuri and Mukundan 2001; Liao et al. 2004; De Oliveira and Crocomo 2009; Singh et al. 2009; Singh and Sood 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 *in* vivo (Ref). Percentage survival is often used as a measure of this. De Oliveira and Crocomo (2009) reported that it is dependent on shoot quality. Similarly, they reported that the acclimatization of aloe plants was better for larger ones. Therefore, during *in vitro* culture, the employment of treatments which guarantee the production of larger shoots is extremely important for further development of micropropagated plantlets in the greenhouse (Ramirez-Malagon et al. 2001).

Activated charcoal (AC) is largely used *in vitro* (Thomas 2008) with the aim to improve growth, multiplication, elongation and rooting of shoots before their establishment *in vivo* (Hemphill 1998; Gubbuk and Pekmezci 2006; Quoirin et al. 2001; Chen 2009). 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 hormonal levels in the culture substrate (Van Winkle and Pullman 2005), alteration of medium hydrogen ion concentration (pH) (Owen et al. 1991) and modification of nutrient availability through cation adsorption (Eymar et al. 2000; Van Winkle et al. 2003; Van Winkle and Pullman 2003). Despite the numerous research reports the role of AC in a culture medium is not completely comprehended. The addition of AC in the culture medium of *A. barbadensis* improves the height, fresh weight and root number of micropropagated shoots (Hashem, Abadi D et al. 2008; Borgognone et al. 2010; Hashem, Abadi D and Kaviani, 2010) and also ensures a better plant growth during acclimatization (Borgognone et al. 2010). There are no scientific reports detailing the underlining reasons why AC promotes the growth of *A. barbadensis in vitro*.

Some authors showed the effect of culture medium pH during *in vitro* development of plants and found that the pH response was species and tissue dependent (Leifert et al. 1992; Ebrahim and Ibrahim 2000). Owen et al. (1991), (Shibli et al. (1999) reported that most plant tissue culture media are poorly buffered and that the pH changes after heat sterilization and during explant culture. This change can affect plant growth as it influences both nutrient and plant growth regulator uptake by regulating their solubility in the culture medium (Bhatia and Ashwath 2005). The anion and cation uptake occurs preferentially at acidic and alkaline pH, respectively (George 1993). The uptake of NO3- and NH4+ is markedly affected by pH, as well as other inorganic ions and organic molecules, depending on their polarity (George 1993).

Nevertheless, no data are available concerning the *in vitro* effects of AC on morphological and physiological response and elemental composition 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. pH stabilization and regulation of NH4+ and NO3- uptake may be the reasons of the higher growth performance of the shoots observed in presence of AC in the medium.

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), NH4+ and NO3-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 were micropropagated on MS (Murashige and Skoog 1962) medium including vitamins (4.4 g l-1) to which sucrose (30 g l-1), Benzyl adenine [(BA) (2.0 mg l-1)], Indole 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 (0.1 N) HCl or NaOH. After 45 days in the medium, new shoots (which had developed) were isolated and placed on one half strength MS medium devoid of growth regulators?? but containing 30.0 g l-1sucrose and 7.0 g l-1 agar to promote elongation and rooting. Two combined factors were tested during *in vitro* elongation and rooting: (i) the presence or absence of 1% (w/v) AC (Duchefa, Haarlem, The Netherlands) in the medium; (ii) 20 and 40 day culture periods. Into each vessel (specify type) 200 ml aliquots of medium were decanted. Nine shoots per vessel were implanted into each culture vessel and transferred to a growth chamber maintained at 24±1°C under a 16 hour photoperiod provided by cool-white fluorescent lamps emitting a photon lux 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 at 121°C and 105 kPa for 20 min.

*Measurements and analysis*

Values of pH and EC of the medium were measured immediately after sterilization using a pH meter (GLP21, Crison Instruments, Barcelona, Spain) and an EC meter (HI 86304, [Hanna Instruments](http://www.hannainst.com/usa/news.cfm?Id=63), 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 was determined for each implanted shoot. Dry weights were determined after drying for 48 hours 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 weight minus the initial weight divided by the initial weight.

At the end of the elongation and rooting period the remaining medium in each vessel was collected to determine pH, EC,Was this measured using the gel form or had it been centrifuged before measurement) and the content of NO3- and NH4+. Nitrate and ammonium concentrations were analyzed in the liquid fraction of substrate which was obtained by the media centrifugation for 20 minutes at 15000 × *g* and filtration through Whatman filter. Nitrate and ammonium concentrations in the liquid fraction of the media 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?the 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).

Five shoots per replicate were oven-dried at 80°C till constant weight and then 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). 0.5 g of the dried tissues were analysed for the following major and trace elements: 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 HNO3 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 (major elements) or μg (minor elements) per g of dry weight.

*Statistical analysis*

All data were analyzed by using the SPSS software package (SPSS 16.0 for Windows). Data were subjected to two-way analysis of variance (ANOVA). Percentage values of dry matter were transformed arcsine 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 = 0.05).What about correlation

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*Growth parameters*

Addition of AC to the culture medium significantly increased shoots length, shoots fresh weight and the elongation rate. The length of the culture period significantly affected shoots length, shoots fresh weight, shoots dry weight and root number. After 40 days of culture shoots length, shoots fresh weight and root number were higher than after 20 days while shoots dry matter was lower at 40 days of culture (Table 1).

Growth index was significantly affected by the interaction between AC and days of culture; the highest GI value was obtained after 40 days in the presence of AC (Fig. 1). The same value of GI was recorded after 40 days of culture without AC and after 20 days of culture with AC. The lowest GI value was recorded after 20 days of culture in the medium without AC.

Also the root to shoot ratio was significantly affected by the interaction between the experimental factors (Fig. 2). 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 without AC was significantly higher than those measured for the treatment with AC. In presence of AC in the medium the root to shoot ratio decreased passing from 20 to 40 days of culture while the opposite trend was observed for the control without AC.

*Medium pH and 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 buffering effect on pH (ranging from 5.71 to 5.42) while the pH values dropped to a value lower than 4.5 in the medium lacking of AC (Tab. 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). The EC value in the medium was affected by the treatment with AC, in fact it was significantly lower in the medium with AC compared to the control without 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.

Both the AC treatment and the length of the culture had a significant effect on ammonium to nitrate uptake ratio (Tab. 2). In fact, this ratio was significantly lower for the treatment with AC respect to the control without AC, and it was higher after 20 days than after 40 days of culture.Looks the other way to me.

After 20 days of culture, there was a significant linear relationship between ammonium to nitrate uptake ratio and medium pH; the increase of the NH4+/NO3- ammonium to nitrate uptake ratio corresponded to a reduction of the medium pH (Fig. 3).Fig 4??

*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 more N than plants cultivated in absence of AC. The total nitrogen content in plant tissues was significantly higher after 40 days of culture than after 20 days (Table 3).

The content of the major as well as of the trace elements in the shoots was affected by the presence of AC in the medium and the culture time. A general decrease of nutrient concentration induced by AC was observed (Table 3).

K content was higher in shoots cultured in absence of AC and it decreased between 20 and 40 days of culture.

P content was affected by the interaction between AC treatment and the days of culture (data not shown); the highest value of P concentration was detected at 20 days of culture in absence of AC (9.26 mg g-1 dw) while the lowest one was recorded after 40 days of culture in absence of AC (5.73 mg g-1 dw). In presence of AC P concentration did not change over the culture period (7.06 and 7.03 mg g-1 dw after 20 and 40 days of culture respectively).

Also, Mg concentration was affected by the interaction between the two experimental factors (data not shown). In fact, Mg concentration decreased after 40 days of culture on medium lacking in AC passing from 2.12 mg g-1 dw at 20 days to 1.73 mg g-1 dw at 40 days. Mg concentration did not change during the culture period in presence of AC (1.83 mg g-1 dw at 20 days and 1.91 mg g-1 dw at 40 days).Again where is this data in Table 3

Ca content was significantly higher in shoots cultured on medium without AC than with AC (Table 3).

The Na concentration in aloe tissues was affected by the interaction between AC treatment and days of culture (data not shown); in fact, in presence of AC the Na content was 1.15 mg g-1 dw after 20 days of culture and it increased to 1.51 mg g-1 dw after 40 days. In absence of AC the Na concentration was 1.49 and 1.53 mg g-1 dw after 20 and 40 days of culture, respectively.

Mn, B and Zn were significantly higher in tissues not treated with AC and Zn increased over the culture period regardless of the medium treatment (Table 3). Also Cu concentration in the tissues increased passing from 20 to 40 days of culture but no effect of the AC treatment was observed (Table 3).

Fe concentration was affected by the interaction between the two experimental factors (Fig 3). In fact, an 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.

**Discussion**

AC is commonly employed in plant tissue culture to improve proliferation, growth and rooting of explants (Thomas 2008). Charcoal is produced by destructive distillation 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 charcoal 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 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 height 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: the best result was achieved by including AC [0.2 % (w/v)] 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).

The 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). Plants under environmental stress easily accumulate more biomass in the roots. In herbaceous mimosa, both root to shoot weight ratio and root to shoot length ratio were affected by soil pH conditions; plants allocated more biomass distribution to the shoots in optimal pH condition and more to the roots under pH stress (Nuruddin and Chang 1999). The conclusion is that AC had a role in setting optimal conditions for *in vitro* elongation and rooting and in reducing stress factors of the *in vitro* environment which may lead to growth inhibition and physiological disorders. Plants *in vitro* are exposed to high osmoticity of the medium, abnormal mineral nutrition, unusual hormonal treatments, high relative humidity of the flask atmosphere and accumulation of gases (ethylene in particular) (Hazarika 2006), leach of phenols into the medium (Krishna et al. 2008) and pH changes (Owen et al. 1991; Shibli et al. 1999). Often the medium pH drifts to an acidic range following the culture period. This could be due to the cations uptake or the predominant uptake of NH4+ with respect to NO3- ions, secretion of organic acid from the plant material, dehydration of media and/or precipitation of medium components (Leifert et al. 1992; Shibli et al. 1999).

The results identified that the main effect of AC exerts 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 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 provided by the MS medium (Eymar et al. 2000).

Some authors reported an effect of AC in preventing the acidification of the medium after autoclaving (Eymar et al. 2000) or even in inducing its slight alkalinisation (Owen et al. 1991). In our experiment the detected values of pH did not suggest any interaction between the addition of AC and the autoclaving, on the contrary the pH slightly increased only in the medium lacking in AC.

There was a negative correlation between NH4+ to NO3- uptake ratio and pH (Fig 4). This finding the role of assimilation of the two nitrogen forms assimilation on buffering the medium pH. It also suggested a possible explanation of the growth promotion observed with AC. The uptake of NH4+ ions by plant cells leads to a rapid shift towards acidity since for each equivalent of NH4+ incorporated into organic matter, about 0.8-1 H+ equivalents are released into the external medium. On the contrary, for each equivalent of NO3- assimilated, 1-1.2 proton equivalents are removed from the medium, inducing an increase of medium pH (George 1993). Therefore, the final pH of the medium depends on the proportions of NH4+ and NO3- which are provided to the culture.

The ratio between the uptake of NH4+ and NO3- was significantly lower in the treatment with AC and the pH of the medium did not decrease with respect to the post-autoclave value. In absence of AC, the NH4+ to NO3- uptake ratio was higher and the medium pH dropped. The results showed that AC increased the assimilation rate of NO3-. During the initial period in culture the higher uptake of NO3- of shoots in presence of AC may have counteracted the lowering of medium pH led by NH4+ uptake. A similar effect of AC has been previously reported in *in vitro* culture of *Lagerstroemia indica* (Eymar et al. 2000).

For some cultures the balance between the two nitrogen forms in the MS medium (ratio NH4+ to NO3-; 34:66) is not optimal (George 1993). The adsorption of NH4+ on the particles surface of AC (Asada et al. 2006; Vassileva et al. 2008) may have led to an improvement of the nitrogen composition of the medium. The proportion of the two nitrogen sources for an optimal performance of plants *in vitro* is species- and tissue-dependent. Grimes and Hodges (1990) demonstrated that plant regeneration and growth from immature embryos of *Oryza sativa* L. is strongly influenced by very small variation of that proportion.

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. The reduced shoot growth in absence of AC could be due to a low rate of nitrogen uptake. In C*amellia sinensis* plants Ruan et al. (2007) found a close relation between low growth rate, reduced concentration of total nitrogen in tissues and a scarce absorption rate of NO3-.

The lower content of nitrogen within the tissues could be a direct consequence of the acidification of the medium lacking in AC. Experiments of nitrogen nutrition with *Picea* *abies* seedlings led us to hypothesize that the presence of NH4+ and the low pH of the nutrient solution decreased the assimilation of NO3- and inhibited the nitrate reductase activity, the key enzyme of nitrogen metabolism in plants (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 NH4+ ions. The hypothesis suggested is that *A. barbadensis* shoots request a lower NH4+/NO3- ratio than that provided through MS medium to better assimilate nitrogen and therefore rapidly grow.

However, despite the significant buffer effect evidenced by the experiments, the involvement of other factors cannot 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 elemental 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 in AC (Fig. 3) 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).

Over the culture time EC decreased with respect to the initial value in both treatments although the decrease was more pronounced in presence of AC. EC may have been decreased by the effect of the AC ability to retain organic and inorganic molecules on its surface.

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. To properly clarify the mechanism course of AC action and its effects on pH, NH4+ to NO3- ratio in the medium and shoot stress status further investigation is required.

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Figure 1. Combined effects of activated charcoal (AC) and days of culture on growth index

Figure 2. Combined effects of activated charcoal (AC) and days of culture on root to shoot ratio

Figure 3. Combined effects of activated charcoal (AC) and days of culture on Fe concentration in *Aloe barbadensis* shoots

Figure 4. Correlation analysis between ammonium to nitrate uptake ratio and medium pH after 20 days of culture in presence or absence of activated charcoal (AC) in the elongation and rooting medium of *Aloe barbadensis* shoots