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ORIGINAL PAPER

Biochar successfully replaces activated charcoal for in vitro culture of two white poplar clones reducing ethylene concentration

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Abstract Biochar (BC) is a carbon rich product resulting from the biomass pyrolysis process and there have been no reports until now on BC effects in tissue cultures as a suitable substitute for activated charcoal (AC). The results of an experiment on two clones of white poplar (*Populus alba* L.) grown in culture media with different amounts of BC (0, 0.5 and 1.5 g/dm^3) showed that its addition did not damage the plants and there were no significant differences comparing the data obtained for the same concentrations of AC. Both BC and AC addition was shown to increase root dry biomass and number of roots per shoot and these effects appeared to be independent of genotype and concentrations of the added products. A greater elongation was also recorded for shoots grown on a substrate containing BC than those grown on media without BC. These effects did not seem to be caused by darkening due to the addition of BC as there are no significant differences between the temperatures of the different culture media, but are probably due to the adsorption of molecules such as ethylene. Indeed, during the experiment, the hormone concentration in the atmosphere was lower in vials containing the media with BC than the BC-free ones after 14 and 21 days: the lower amount of ethylene in the medium with BC could explain the difference in shoot elongation and the abundant root biomass since high ethylene concentration could inhibit organogenesis.

Keywords Black carbon · Effects · Temperature · Ethylene · *Populus alba* L.

Abbreviations

AC	Activated carbon
BC	Biochar
QRC	Querce clone
VIL	Villafranca clone
WPM	Woody plant medium

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Introduction

Activated carbon (AC) is manufactured by heating carbonaceous material at a high temperature (above 500 °C) over long periods of time (>10 h) and finally activated with energy wasteful processes. AC, produced from wood, wood waste, paper-mill waste liquors and peat (El-Hendawy et al. 2001) is often used in plant tissue culture: it is well known that it may improve cell growth and development (Pan and Van Staden 1998) and better growth responses of plant tissues are generally associated with the addition of AC to both liquid and semi-solid media (Anagnostakis 1974; Fridborg and Eriksson 1975; Horner et al. 1977; Weatherhead et al. 1978; Peck and Cumming 1986; Bon et al. 1988; Zaghmout and Torello 1988). AC has been

used to promote somatic embryogenesis (Buccheim et al. 1989; Andrade and Merkle 2005; Zouine et al. 2005), androgenesis (Johansson 1986; Nomizu et al. 2004; Laura et al. 2006), rooting (Sul and Korban 2004; Loc et al. 2005), shoot and root elongation (Mohamed-Yasseen 2001; Sul and Korban 2005), bulb formation (Peck and Cumming 1986; Vishnevetsky et al. 2003) and to inhibit tissue browning (Mohamed-Yasseen 1994; Mohamed-Yasseen et al. 1995). For an extensive review on the effects of AC in tissue culture, see e.g. Thomas (2008). These effects may be due to various reasons, e.g. the darkening (Pan and Van Staden 1998) and aeration of culture media (Dumas and Monteuuis 1995; Mohamed-Yasseen 2001), the release of substances naturally present in AC which promote growth (Misson et al. 1983) and the adsorption of certain molecules which later become available to plants (Johansson and Eriksson 1977; Johansson et al. 1990; Fridborg et al. 1978). However these mechanisms are largely unclear. Johansson et al. (1982) and Johansson (1983) demonstrated that the stimulatory effect of AC on plant growth may be caused by the adsorption of plant hormones, phenolic compounds and/or inhibitory substances from the nutrient solution, such as 6-benzyladenine and, in particular, ethylene. Ethylene, a gaseous plant hormone that can inhibit shoot elongation (Guzmán and Ecker 1990), has been well studied in relation to vegetative plant growth, seed dormancy and germination processes (Abeles et al. 1992), but only few studies have focused on the relationships between charcoal, ethylene and those physiological processes. In a study on *Nicotiana tabacum* L., Horner et al. (1977) proposed that AC could be able to adsorb ethylene released by the medium and by plant nodes, reducing its accumulation in closed culture vessels and thus increasing growth.

One very interesting option in tissue culture may be the substitution of AC with biochar (BC), a carbon-rich material similar in appearance to charcoal, produced by pyrolysis. The term BC was originally associated with a specific type of production, known as 'slow pyrolysis', a type of pyrolysis in which oxygen is absent, heating rates are relatively slow and peak temperatures relatively low. However, the term BC has been extended to products of short duration pyrolysis at higher temperatures known as 'fast pyrolysis' or to products of pyrolytic stoves. Due to its chemical structure, BC exhibits a long mean residence time in soil, estimated between 1,000 and 10,000 years (Lehmann et al. 2008; Liang et al. 2008; Kuzyakov et al. 2009; Major et al. 2010). Given this recalcitrance, BC is beginning to receive attention as a potential soil carbon sequestration method (Lehmann 2007) and has recently been proposed as a promising management option to reduce atmospheric CO_2 concentration (Smith et al. 2010). Moreover, the BC application to soil improves its physical and chemical characteristics (Chan et al. 2007) and hydraulic properties (Lehmann and Joseph 2009; Woolf et al. 2010). Many studies have recently shown the positive effect of the addition of BC to soil on field crop growth (Lehmann et al. 2003; Lehmann and Rondon 2006; Yamato et al. 2006; Chan et al. 2007; Rondon et al. 2007; Kimetu et al. 2008; Sinclair et al. 2008; Steiner et al. 2008; Van Zwieten et al. 2008; Blackwell et al. 2009; Baronti et al. 2010; Gaskin et al. 2010; Noguera et al. 2010; Vaccari et al. 2011). Furthermore, it has been proved that BCs are characterized by high affinity for organic contaminants (Cao et al. 2009; Kookana 2010; Wang et al. 2010; Sun et al. 2011a, b) and heavy metals (Beesley and Marmiroli 2011) because of their adsorptive capacity, thus in other contexts these materials may potentially be an alternative for AC (Oleszczuk et al. 2012). No data are reported for the use and effects of BC in in vitro cultures.

This paper describes the effects of the in vitro inclusion of BC in the medium of two shoot cultured clones of white poplar (*Populus alba* L.), in comparison with the traditional use of AC. Particular attention has been paid to evaluating the interactions among root and shoot growth, medium temperature, which could be affected by the dark color of BC and AC, and ethylene accumulation in the vial atmosphere, in order to ascertain some possible action mechanisms of BC on cultured shoots.

Materials and methods

Plant material and experimental conditions

In vitro proliferating shoots of a commercial and an autochthonous *Populus alba* L. clone, Villafranca (VIL) and Querce (QRC), respectively, were subcultured on woody plant medium (WPM; Lloyd and McCown 1980). Media contained 20 g/dm^3 of sucrose and 0.2 mg/dm^3 of benzyladenine and were solidified with 7.2 g/dm^3 of agar (B&V, Reggio Emilia, Italy), at pH 5.5, in 100 cm^3 baby-food glass jars (Sigma-Aldrich, Milano, Italy). Media were sterilized by autoclaving at 121 °C and 108 kPa for 20 min. Shoots originated from shoot tips collected from in vivo adult trees of the above-mentioned clones were multiplied by axillary bud proliferation, according to Confalonieri et al. (2003), with 4-week subculturing. The jars were incubated in a growth chamber at 23 ± 1 °C with a 16-h photoperiod (light intensity, 40 μmol m^{-2} s^{-1}).

Before the treatments, shoots were transferred to phytohormone-free WPM medium. After four weeks, well-developed shoots were cut to uniform size (two internodes; 1.5 cm tall) and transferred to phytohormone-free WPM medium containing BC (1.5 g [BC1] and 0.5 g [BC2] per

dm^3) and AC (1.5 g [AC1] and 0.5 g [AC2] per dm^3) to evaluate rooting capacity. BC was crushed to a powder before being added to the medium in order to increase the area/volume ratio and have similar size to AC particles (<2 mm). The color of medium with BC was similar to that with AC (Fig. 1). The evaluation of shoots on media without AC or BC (controls or C) was included in the experiment. Twenty plants per clone per concentration replicated three times (four glass jars per treatment per clone) were used in the experiments. After 30 days, the shoots were gently extracted from the medium and separated into shoots and roots. The following parameters were measured: (1) weight and length of shoots and (2) weight and number of roots. Roots and shoots were oven-dried at 80 °C for 24 h after which dry weights were recorded.

Chemical analysis

BC was obtained from poplar biomass using a pyrolitic stove that has a coaxial burner with a swirl pattern, resulting in highly efficient syngas combustion and heat transfer. The temperature of pyrolysis, measured inside the stove with thermocouples (K type, COD. 406-486, TC Direct Srl) inserted in burning poplar biomass, was 550 °C. AC is a commercial product purchased from Carlo Erba Reagenti SpA company (Cod. 7440-44-0).

Chemical analyses were performed on BC and AC (Table 1). C and N contents were determined using a CHN Elemental Analyzer (Carlo Erba Instruments, mod 1500 series 2). The dry samples were acid-digested in a microwave oven (CEM, MARSXpress) according to the EPA method 3052 (USEPA 1995). The solutions obtained after mineralization were filtered with 0.45 μm PTFE and then diluted. Macro and microelements were determined by acid digestion of 0.05 g in 5 cm^3 of nitric acid (HNO_3); after 30 min, 2 cm^3 of perchloric acid ($HClO_4$) were added according to Miller (1998). The digested samples were first centrifuged and then filtered through a ceramic filter. The samples were subsequently analyzed with an ICP-OES spectrophotometer (IRIS Intrepid II XSP Radial, Thermo Fisher Scientific). The pH values were measured in water solution (1:4).

Table 1 Characteristics of BC and AC used

Properties	BC	AC
Total C (%)	78.69	81.06
Total N (%)	0.11	0.34
P (mg kg^{-1})	405.2	189.5
K (mg kg^{-1})	1584.0	742.4
Ca (mg kg^{-1})	7534.0	7649.0
Mg (mg kg^{-1})	714.0	614.03
pH (1:4 H_2O)	7.0	7.2

Measurements of culture media temperature

The temperature of the culture media was measured by using 1.5 mm diameter-micro-thermistors (Ntc, negative temperature coefficient—thermistors of 10 kOhm; PR503J2, US Sensor Corp.) with low thermal inertia and fast time response. The thermistors were previously cross-calibrated and soaked for 20 min in 1.4% sodium hypochlorite solution and then used to detect the temperature of three different culture media in three different jars: (1) WPM; (2) WPM with 1.5 g/dm^3 of BC added (BC1); (3) WPM with 0.5 g/dm^3 of BC added (BC2). Each 100 mm diameter glass jar contained three micro-thermistors connected to a data-logger (mod. Delta-T DL2, Delta-T Devices, UK) for 2 days; all temperature data were collected with a time acquisition interval of 10 min.

Determination of ethylene production

In vitro shoot cultures of VIL clone were used as source of explants, consisting of 1.5-cm shoots with two internodes and no roots. After isolation, shoots were transferred to phytohormone-free WPM medium for 2 days in order to remove the ethylene production caused by the cuts during

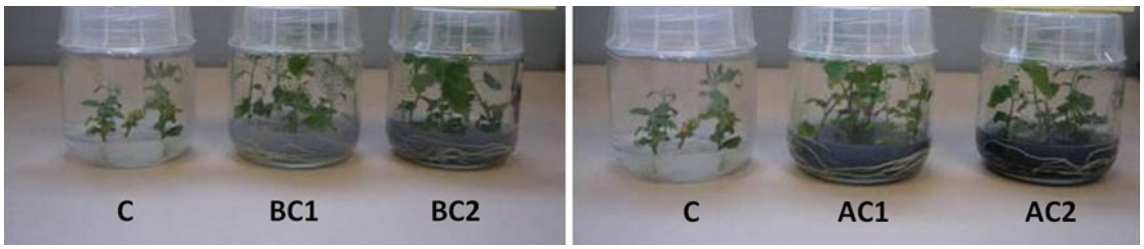


Fig. 1 Shoots of *Populus alba* L. clones (Villafranca) grown on different media after 30 days of culture. C, control; BC1, medium with 1.5 g/dm^3 of BC added; BC2, medium with 0.5 g/dm^3 of BC added; AC1, medium with 1.5 g/dm^3 of AC added; AC2, medium with 0.5 g/dm^3 of AC added

subculturing. The shoots were then transplanted singly into 20 cm^3 vials containing WPM (in the text WPM + P, where P stands for plant) and WPM with 1.5 g/dm^3 of BC added (WPM + P + BC1). Twelve shoots were considered per treatment. Vials with only WPM (WPM) and WPM with 1.5 g/dm^3 of BC added (WPM + BC1) were included in the experiment to evaluate ethylene accumulation in the vial atmosphere. Vials were capped with airtight serum caps (Sigma Chemical Co., St. Louis, MO, USA), in order to completely seal the containers (Joy et al. 1991). All flasks were maintained in a growth chamber at the conditions previously described.

For the determination of ethylene accumulation inside the vials, gas samples (1 cm^3) were withdrawn from the atmosphere of the containers and injected into a Perkin-Elmer Autosystem Gas Chromatograph (Boston, MA, USA), equipped with a flame ionization detector and a Poropak Q column (80–100 Mesh). Column, injector and detector temperatures were 50, 50 and 250 °C, respectively. Nitrogen was the carrier gas at 20 psi. Ethylene concentration was determined in comparison to a standard of 0.01 cm^3/dm^3.

Statistical analysis

Statistical analysis was carried out with one-way and two-way analysis of variance (ANOVA) using the statistical program Statistica 6.0 (StatSoft, 2003). Mean separation was obtained by means of the Tukey HSD test. Clones and treatments were considered as independent variables. Means were considered different at a probability level of $P \leq 0.05$.

Results and discussion

After 30 days from transferring onto culture medium, all shoot cultures had rooted (Fig. 1). The root dry biomass of the two white poplar clone shoots, VIL and QRC, treated with BC and AC at the two different concentrations (0.5 and 1.5 g/dm^3) differed significantly from the control (14.5–18.1 mg against 5.6–7.6 mg of non-treated shoots) (Fig. 2a). No differences were detected between BC and AC treatments nor between the two genotypes ($P \leq 0.05$). Indeed both VIL and QRC responded in a similar way to both treatments (AC and BC) and at different concentrations, since average values of root dry biomass did not result as significantly different (15.8 mg [QRC microshoots in AC1], 14.5 mg [QRC microshoots in AC2], 15.9 mg [QRC microshoots in BC1], 16.4 mg [QRC microshoots in BC2], 16.9 mg [VIL microshoots in AC1], 15.6 mg [VIL microshoots in AC2], 16.3 mg [VIL microshoots in BC1] and 18.1 mg [VIL microshoots in BC2]) (Fig. 2a).

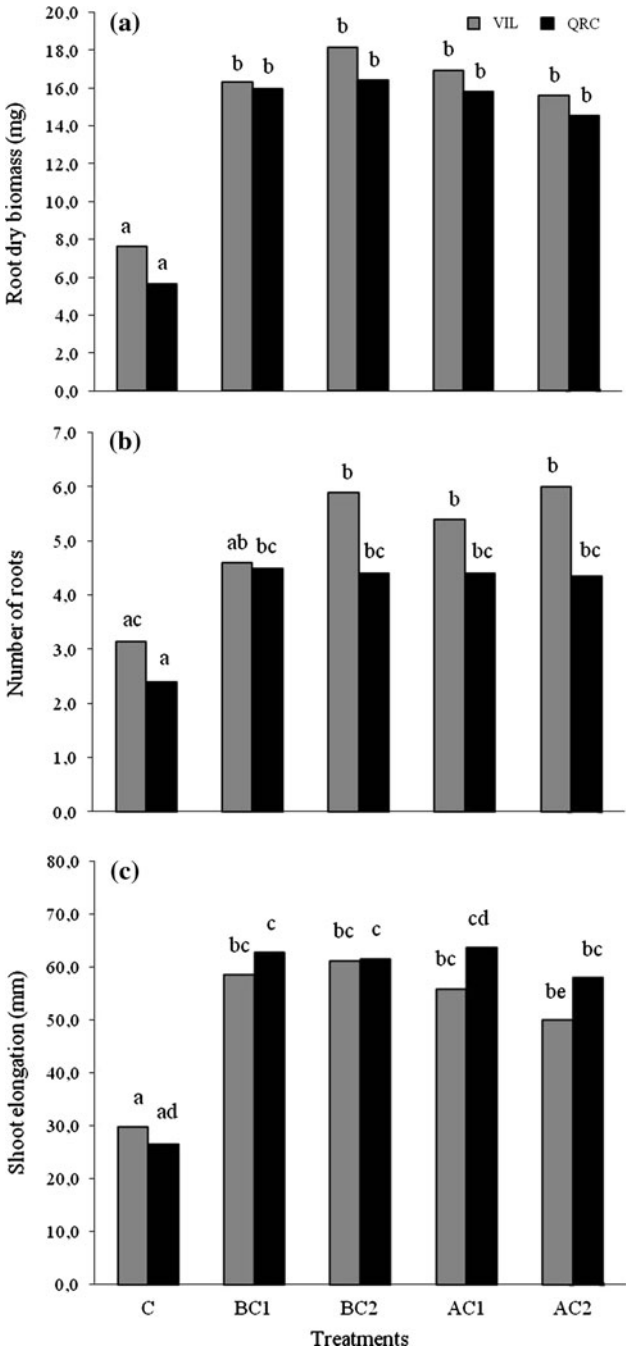


Fig. 2 Root dry biomass (mg) (**a**), number of roots (**b**) and shoot elongation (mm) (**c**) of *P. alba* clones after BC and AC treatments. Values are means of at least twenty values; significant differences between the means (at least $P \leq 0.05$, according to ANOVA) within one clone appear with different letters. C, control; BC1, medium with 1.5 g/dm^3 of BC added; BC2, medium with 0.5 g/dm^3 of BC added; AC1, medium with 1.5 g/dm^3 of AC added; AC2, medium with 0.5 g/dm^3 of AC added; VIL, Villafranca clone; QRC, Querce clone

Whereas no significant differences were detected for shoots in any of treatments (data not shown).

A positive effect of AC and BC was also detected on the rootability of shoots, regardless of the concentration in

almost all treatments (Fig. 2b). It resulted in a significant increase in number of roots; indeed shoots of both clones growing on media with AC or BC produced more roots (4–6) on average than the control (2–3) ($P \leq 0.05$). A similar effect was reported by Dumas and Monteuuis (1995) for juvenile *Pinus pinaster* L. explants cultured in the presence of AC. Influence on rooting was also reported by Firoozabady et al. (2006); in their work the addition of AC considerably enhanced the rooting ability of transgenic shoots in pineapple.

AC was also found to induce shoot maturation and elongation, as observed in cotton (*Gossypium hirsutum*) cultured on Murashige and Skoog medium fortified with 3 g/dm^3 of AC (Hemphill et al. 1998) and in corn shoots cultured in a medium with 5 g/dm^3 of AC and [^{3}H] marked gibberellin (Mohamed-Yasseen 2001).

Data in Fig. 2c show that in general both AC and BC treatments enhanced shoot length by about 100% ($P \leq 0.05$) for QRC in BC1 (27 mm of C vs. 63 mm of BC1), in BC2 (27 mm of C vs. 61 mm of BC2), AC1 (27 mm of C vs. 64 mm of AC1) and AC2 (27 mm of C vs. 58 mm of AC2) even if shoot dry biomass was not significantly different (data not shown). The length was always greater than the control ($P \leq 0.05$) for VIL plants grown on media with BC1 (30 mm of C vs. 59 mm of BC1), BC2 (30 mm of C vs. 61 mm of BC2), AC1 (30 mm of C vs. 56 mm of AC1) and AC2 (30 mm of C vs. 50 mm of AC2).

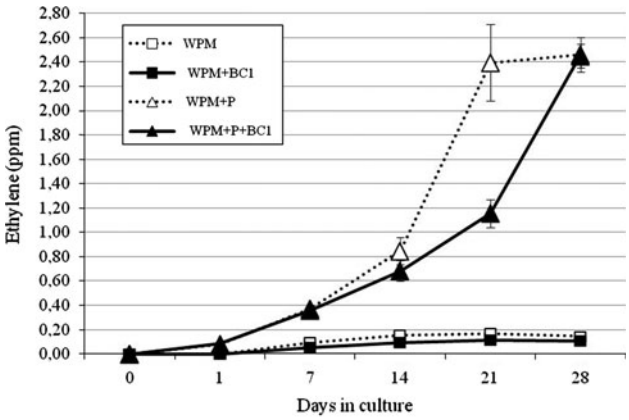
The overall average temperatures measured by the micro-thermistors inserted directly in the media without shoots are indicated in Table 2. No significant differences were detected among the three tested media throughout the experimental period. We can therefore assume that the grey color of the medium did not affect the temperature.

White poplar shoots produced ethylene which accumulated in the culture vials (Fig. 3). The maximum mean amount observed was about 2.46 ppm after 28 days from the beginning of the experiment. No initial burst of ethylene was detected but there was a relevant rise in the amount produced from the 7th day associated with the rapid growth of the shoots (data not shown).

Table 2 Means of temperatures of three different culture media

Treatment	°C
WPM	23.2 ± 0.43 a
WPM + BC1	23.2 ± 0.43 a
WPM + BC2	23.1 ± 0.42 a

Significant differences between the means (at least $P \leq 0.05$, according to ANOVA) appear with different letters. WPM, woody plant medium, WPM + BC1, woody plant medium with 1.5 g/dm^3 of BC added; WPM + BC2, woody plant medium with 0.5 g/dm^3 of BC added



Day in culture	WPM	WPM+BC1	WPM+P	WPM+P+BC1
0	a	a	a	a
1	a	a	b	b
7	a	a	b	b
14	a	a	b	c
21	a	a	b	c
28	a	a	b	b

Fig. 3 Ethylene content (ppm). Values are mean ± standard error; significant differences between the means (at least $P \leq 0.05$, according to ANOVA) appear with different letters in every row of the enclosed table. *Open square*, *WPM* woody plant medium; *filled square*, WPM + BC1, woody plant medium + 1.5 g/dm^3 of BC; *open triangle*, WPM + P, woody plant medium + plant; *filled triangle*, WPM + P+BC1, woody plant medium + plant + 1.5 g/dm^3 of BC

The addition of BC1 to the culture medium markedly influenced ethylene concentration in the shoot-containing vials. Indeed, in the central period of subculture (from day 14 to day 21) a significant reduction of ethylene accumulation in the atmosphere of vials containing shoots on WPM + BC1 was observed, in comparison with the shoots that were cultured on BC-free medium. It is possible to hypothesize the adsorption of ethylene by BC, as already reported for AC that seemed not only to adsorb ethylene (Mensuali-Sodi et al. 1993; Horner et al. 1977; Fridborg et al. 1978) but also other substances e.g. growth regulators and inhibitory molecules (nicotinic acid, Weatherhead et al. 1978; iron chelate and folic acid, Johansson et al. 1990). We can also suppose that BC is similar to AC because the ethylene curve was similar to Mensuali-Sodi et al. (1993), even if the paper was on AC. No report exists about inhibit growth regulators/inhibitory molecules and BC but many papers report that BC could adsorb molecules such as pesticides (for this topic, see e.g. Hale et al. 2011; Sun et al. 2011a, b; Song et al. 2012; Sopeña et al. 2012; Sun et al. 2012a, b; Uchimiya et al. 2012) and heavy metals (see e.g. Park et al. 2011; Borchard et al. 2012). Of course it has been reported that the effect of AC on ethylene adsorption is dependent on culture conditions, such as container volume and shape, medium volume and surface exposed to the inner atmosphere (Mensuali-Sodi et al. 1993) but in the present study ethylene decrease was also evident in 20 cm^3 vials that had a low agar surface. The low BC quantity in the

medium also seemed to account for a time-limited adsorption of the gaseous hormone, as ethylene concentration was similar after 28 days in both the BC-containing and BC-free vials. Vials with only WPM (WPM) and vials containing WPM with BC added (WPM + BC1) showed no ethylene accumulation, differently from what was observed by Spokas et al. (2010) who also found ethylene production analyzing sterile soil amended with BC. Ethylene production has been observed in some types of biochar (Spokas et al. 2010) and this only apparently contradicts the results reported in this study, where ethylene absorption was instead detected. Such a discrepancy may in fact be reconciled considering that the ethylene production reported by Spokas et al. (2010) was only associated to non-woody feedstocks and decreased with increasing pyrolysis temperature above 400 °C since the properties of the resulting biochar vary as a function of the feedstock and conditions of the pyrolysis (Sensöz 2003; Guerro et al. 2005). This suggests the co-occurrence of ethylene emission/absorption capacity in biochar and the fact that in some types of biochar produced at high temperature and from wood feedstocks the latter effect definitely prevails. This is of importance for in vitro cultures where high concentrations of this gas may have negative effects on plant organogenesis since high ethylene concentration could inhibit organogenesis (Guzmán and Ecker 1990). Moreover, recent studies (Spokas et al. 2011) reported that a wide range of organic volatile compounds (VOCs) are released by biochar and the hypothesis that some of those VOCs may have an effect on growth cannot be ruled out (Simms and Rausher 1987; Ryu et al. 2003; Kloepper et al. 2004; Baldwin et al. 2006; Zhang et al. 2007).

Conclusion

The data reported in this paper can have practical importance for future applications of BC in in vitro cultures. Our results showed that the charcoal concentrations for both AC and BC (0.50 and 1.50 g/dm^3) have the same effects on poplar tissue culture since no major differences were found between the two types of treatment. No negative effects of BC were detected for white poplar shoot cultures while the addition of AC or BC was shown to improve the potential for rooting in terms of enhancement of the dry biomass as well as the number of roots and these effects appeared to be independent of genotype and concentrations used in our experiments. There was no darkening effect of BC on culture media temperature so this is probably not the factor that enhanced shoot and root growth and development. No ethylene emission was found in vials containing only WPM and BC, and lower ethylene concentration after 14 and 21 days in vials with shoots grown on WPM with BC added than controls. But those results require extensive validation before the use of BC can become accepted practice. The identification of the most effective BC types and their subsequent standardization are certainly the most critical aspects that must be properly addressed. Even BC made from the same type of feedstock can in fact be very different depending on the production conditions as well as on the quality of the biomass (Uchimiya et al. 2011). The advent of new biomass gasification technologies that produce BC in a standardized process (De Pasquale et al. 2012) can certainly contribute towards adequate standardization, but the commercialization of BC stocks suitable for routine in vitro culture techniques will require the use of simple but robust analytical procedures to assess its mean porosity and the associated variability. Nitrogen adsorption tests and electron microscopy have already been successfully used to determine BC porosity (Sun H et al. 2012a, b) and Fast Field Cycling Nuclear Magnetic Resonance Relaxometry (FFC-NMR) is also very promising (Anoardo et al. 2001). We can conclude that (1) BC can be used in in vitro cultures at least for white poplar and can therefore replace AC which is certainly a much more expensive product, once biochar with a uniform quality can be guaranteed, (2) the feedstock and pyrolisis temperature are of importance in biochar production if ethylene absorption is required and (3) potential interactions must be elucidated between VOCs emissions and plant growth in in vitro cultures using biochar.

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