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Influence of microstructured carbon materials on *Sequoia sempervirens* (D. Don.) Endl. *in vitro* culture

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Abstract. The present experimental research has been mainly focused on *Sequoia sempervirens* (D. Don.) Endl. as woody plant model system, for testing the influence of some microstructured carbon materials (CCM) comparing with the activated charcoal action, on the *in vitro* growth. The work hypothesis was that CCM can induce effects on the plant culture, similar to those induced by activated charcoal, due to their high absorption capacity. The influence of two types CCM and activated charcoal introduced in the medium culture was evaluated on the fresh weight and on the length of the *in vitro* regenerants, after 30 and 60 days of cultivation. Our data revealed that the CCM is not toxic and does not inhibit plant growth. We also remarked that *S. sempervirens* (D. Don.) Endl. was more reactive to the activated charcoal into the nutritive medium than the CCM compounds. The use of these new microstructured compounds is an important step both in the study of materials science and in the field of plant biotechnologies
Keywords: *Sequoia sempervirens*, microstructured carbon materials, *in vitro* culture

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Introduction

It is widely recognized that forests are the most biologically diverse terrestrial ecosystems and that pressure on forest biodiversity continues to increase throughout the world (Palmberg-Lerche et al. 2002). Since 1930, a lot of progress has been made in the culture of tissues, organs, cells and protoplasts of woody plants. That is not to say that all woody plants, including forest trees, can be induced to grow and differentiate *in vitro*. Some of them do it, while others are still recalcitrant (Ahuja 1993). Activated charcoal, also called activated carbon or activated coal, it is a form of carbon that has been processed to make it extremely porous and thus to have a very large surface area available for adsorption or chemical reactions (Anonymous).

In the plant cell culture, the activated charcoal is used to assure the absorption of organic and inorganic components at the nutritive medium level, the absorption of impurities at the agar level, the absorption of some catabolists with inhibitor effect on growth processes and their liberation, which it has a

stimulative effect (David et al. 1979). In the same time, the activated charcoal can assure the optical density necessary for the medium, pH effects, the absorption of compounds secreted during the sterilization from the zaharose degradation and the absorption of some toxic compounds resulted from oxidative and metabolic processes (Cachita et al. 2004). Especially in the case of woody plants, activated charcoal bound to phenolic compounds and tannins (responsible for the necrosis of tissues inoculated on aseptic medium) and in this case do not inhibit the plant tissue (Margara 1982). Activated charcoal has a substantial role in the process of *in vitro* buds elongation on aseptic medium, in case of *Sequoia* sp. (Boulay et al. 1979). The present experimental research is using *Sequoia sempervirens* (D. Don.) Endl. (Fam. Taxodiaceae), a native species on the west coast of North America (Korban & Sul 2007). It is also cultivated in Romania (Cluj, Arad, Caraş Severin, Bihor, Hunedoara - Arboretum Simeria and Bucharest) in parks and botanical gardens. The research has been mainly focused on this species as a woody plant model system, for testing the influence of some microstructured carbon materials (CCM) comparing with the influence of the activated charcoal on *in vitro* growth. CCM consist of plane carbonic grids interleaved with layers of intercalated atoms/substances. Based on the intercalated species and on the intercalation method, the host substrate is modifying the properties, being possible major structural changing (Kang et al. 2002).

The work hypothesis for the addition of these compounds in culture medium starts from the premise that CCM, as high surface area carbon materials, can cause within the plant cell culture similar effects as those induced by the activated charcoal. The same actions promoted by CCM are attributed to their physical, chemical and structural characteristics (Hristea et al. 2005).

Materials and methods

The explants for inoculation consist in long shoots apex of 2.5-3 cm, excised from an *in vitro* stock culture of *Sequoia sempervirens* (D. Don.) Endl., from the collection of tissue culture of Simeria Forest Research Station, obtained by the kindness of Dr. Magdalena Palada. The nutritive medium was represented by a basal medium MS (Murashige & Skoog 1962) added with 0.2 mg/l kinetin (Duchefa Biochemie B.V.) as cytokinines.

The two CCM whence have been tested (named "A" and "B" by the brevet author), proceed from the National Institute for R&D in Electrical Engineering ICPE-CA.

"A" compound represents expanded graphite obtained from an intercalated compound with H_2S0_4, with a surface area of 158 m^2/g.

"B" compound represents expanded graphite obtained from $FeCl_3$ with a surface area of 10 m^2/g. The "A" microstructured carbon compound is mainly micro-and mezoporous, while "B" compound is mostly macroporous. They have been included in the growth medium in concentrations of 2g/l, after medium sterilization.

During the research, four different experimental variants have been analyzed: Control (C), A, B, and CA (Table 1).

The control (C) was represented by the basal nutritive medium Murashige-Skoog (1962), supplemented with 0.2 mg/l kinetin, as phytohormon source.

The experimental variants A, B, CA contain the same nutritive medium as the Control. 2 g/l of "A" compound was added to the A experimental variant. 2 g/l of "B" compound was introduced into the medium of the B experimental variant and the same concentration of activated charcoal in the CA experimental variant.

All the medium variants were solidified using a concentration of 8% Merck agar, the medium was equally allocated in Sigma tube,

Table 1 Types of medium variants used in the experiment

Experimental variants	Medium content
Control (C)	MS + 0.2 mg/l kinetin
A	MS + 0.2 mg/l kinetin + 2g/l compound “A”
B	MS + 0.2 mg/l kinetin + 2g/l compound “B”
CA	MS + 0.2 mg/l kinetin + 2g/l activated charcoal

10 ml/vial.

Culture conditions were represented by two successive growth periods that took place in controlled environment (constant humidity) such as growth chamber type Friocell 404 (produced by BMT Medica Technology, Czech Republic) at 24^{0}C, under a light flow of 57 µmol/m^2/s and 16/8 photoperiod, each growth period lasting 30 days.

At the end of each 30 days of the growing interval, biological parameters such as fresh mass and length, corresponding to each *in vitro* regenerant/experimental variant were estimated.

The fresh mass (mg) of the *in vitro* regenerants was measured by making the difference between the weight of the inoculum at 30 days, respectively 60 days and the initial weight of the *in vitro* regenerant. The weight determination took place in sterile conditions with a 572 precision balance (Kern).

The length (cm) corresponding to each *in vitro* regenerant/experimental variant was estimated making the difference between the stem length of the *in vitro* regenerant after 30, 60 days of growing (measured in sterile conditions with the ruler) and the initial length of the inoculum.

These two biological parameters were determined for each plant/each medium variant at 30, respectively 60 days of the growing period. 11 repetitions were analyzed in this way, for each experimental variant.

Statistical analysis were done using Sigma Plot 11.0 (Systat Software Inc.). The experimental data were tested for normality using the Shapiro-Wilk test. For the data sets that failed the test we used, it was the Kruskal-Wallis One-Way Anova on Ranks. For the normally distributed data we used a One-Way Anova.

Results

The response of *Sequoia sempervirens* (D. Don.) Endl. regenerants to the supplemented CCM and CA is emphasized in the Figure 1(a and b) and in the Tables 2 and 3.

The fresh mass was not normally distributed after 30 days (Shapiro-Wilk test, $p < 0.05$). The differences in the median values among the experimental variants were greater than would be expected by chance (Kruskall-Wallis One-Way Anova on ranks, $H = 9.961$, $d.f. = 3$, $P = 0.019$). The fresh mass after 60 days was normally distributed (Shapiro-Wilk test, $p > 0.1$). The differences in the mean values among the treatment groups are not statistically significant (ANOVA, $F = 2.830$, $d.f. = 42$, $P = 0.051$). (Table 3)

All the compounds supplemented on the medium (CCM, "A" compound, "B" com-

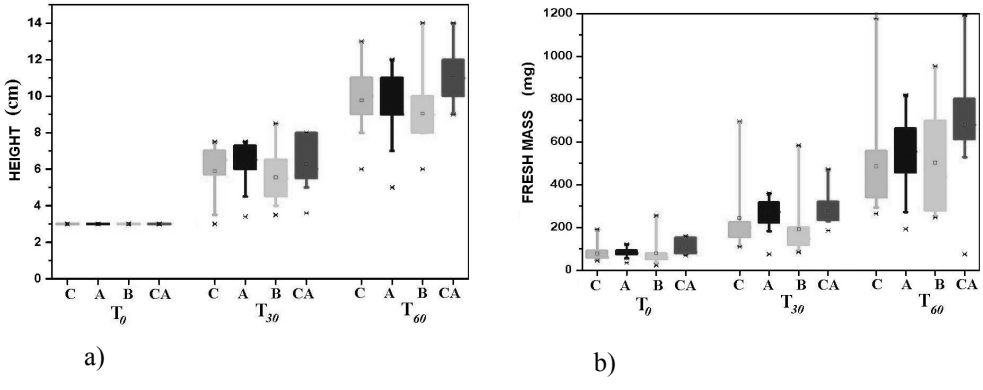

Figure 1 The distribution of regenerant (a) height data (cm) and (b) fresh mass data (mg) based on box plots for each experimental variant, displaying the median (empty square), the likely range of variation (large rectangle), the full range of variation (line ranging from min to max) and outliers (x). Tn indicates the length of the growth period, at the beginning of the experiment (n = 0), and after one (n = 30 days) and two months (n = 60 days).C - control medium variant; A- medium variant containing compound "A"; B - medium variant containing compound "B"; CA - medium variant containing activated charcoal

pound, CA) had a slow stimulative effect, especially in the second growth period.

The same evolution of regenerants could be observed also for the second biological parameter analyzed (height).

Height data were normally distributed at both moments in time (Shapiro-Wilk test, $p > 0.1$). The differences in the mean values among the treatment groups after one month were not statistically significant (ANOVA, $F = 0.643$, $d.f. = 43$, $p = 0.592$), but they were significantly greater after two months (ANOVA, $F = 2.865$, $d.f. = 43$, $p = 0.049$). (Table 2)

Discussion

The research is original, being one of the first trials for testing CCM in case of plant *in vitro* systems.

CCM tested into a different *in vitro* experimental system (*Vitis vinifera* callus culture) has demonstrated a stimulative *in vitro* effect of these compounds on the cell proliferation and on the anthocyanin biosynthesis (Cogălniceanu et al. 2006). These results encouraged us to test these medium supplements on other *in vitro* experimental systems. We tested the influence of these graphite intercalated compounds on the *in vitro* growing processing of *Sequoia sempervirens* (D. Don.) Endl. The experimental data allowed us to ascertain that the specific response of the biological system to the treatment with CCM depends on the species and the experimental system used. Thus *Vitis vinifera* and *Sequoia sempervirens* systems showed a high variability in their *in vitro* responses. Probably the *in vitro* effects of CCM are intermediated by specific mechanisms, different from those corresponding to the activated charcoal. In this way, regarding the *Sequoia sempervirens* (D. Don.) Endl., the positive effects were modest in the first 30 days of growing, but they were strengthen for the second period of growth (60 days). It is important to mention that in all the cases studied by us, these compounds are not toxic and they are not modifying the normal pattern of cytodifferentiation and morphogenesis. For this reason, further studies on the effects

Table 2 Variation of height (length) (cm) of regenerants at different moments for different experimental variants. Mean standard deviation and range (min-Max) are represented (n = 11)

Growth period (days)	Experimental variant			
	C	B	A	CA
T = 0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0
	3.0 - 3.0	3.0 - 3.0	3.0 - 3.0	3.0 - 3.0
T = 30	5.9 ± 1.6	5.5 ± 1.4	6.2 ± 1.3	6.3 ± 1.4
	3.0 – 7.5	3.5 – 8.5	3.4 – 7.5	3.6 – 8.0
T = 60	9.8 ± 1.9	9.0 ± 2.0	9.1 ± 2.0	11.1 ± 1.8
	6.0 – 13.0	6.0 – 14.0	5.0 – 12.0	9.0 – 14.0

Table 3 Variation of fresh mass (mg) of regenerants at different moments for different experimental variants. Mean standard deviation and range (min-Max) are represented (n = 11)

Growth period (days)	Experimental variant			
	C	B	A	CA
T = 0	78.0 ± 41.7	80.3 ± 64.6	77.5 ± 22.8	104.9 ± 36.7
	46.0 - 191.0	24.0 – 256.0	35.0 – 121.0	69.0 – 159.0
T = 30	244.3 ± 176.9	192.3 ± 142.5	257.2 ± 83.5	279.1 ± 79.7
	109.0 – 696	85 – 584	76.0 – 359.0	186.0 – 472.0
T = 60	485.8 ± 287.8	503.0 ± 252.0	533.1 ± 189.6	680.5 ± 276.4
	265.0 – 1284.0	248.0 – 953.0	192.0 – 819.0	76.0 – 1194.0

induced by the graphite intercalated compounds on different experimental biological materials have to be continued and represent an important step both in the study of materials and in the field of biotechnologies.

Conclusions

The results of the current experiment lead to the conclusion that the two CCM are not toxic and reveal a slightly stimulative effect on the normal morphogenetic processes comparing to the Control and they become more accentuated in time.

These data persuade us to continue the research to establish an optimum protocol of multiplication at *Sequoia sempervirens* (D. Don.) Endl. This is more important as *Sequoia* sp. defines the Pacific Coast ecology system and it is in danger of being lost or destroyed by logging, development and environmental pollution in this area (IUCN, 2002). Being a threatened species, any effort in direction of improving *in vitro* protocol for multiplication is justified and could be applied in direction of the "*ex situ*" conservation.

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