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BRIEF COMMUNICATION

The effect of activated charcoal supplemented media to browning of *in vitro* cultures of *Piper* species

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Abstract

With the aim to effectively minimise the browning of tissue cultures of different *Piper* species (*P. longum, P. attenuatum, P. betle, P. nigrum*) explants from stem (nodal and internodal segments), petiole, and leaf were planted on Murashige and Skoog's basal medium supplemented with activated charcoal (AC). AC in the concentration of 200 mg dm^{-3} proved to be the best. Among the taxa studied, *P. longum* showed the least browning whereas, wild *P. nigrum* showed maximum browning.

Additional key words: phenolics, plant regeneration.

One of the major problems associated with plant tissue culture is browning of the culture medium and the explants, which invariably leads to the death of the plants. In order to control browning of medium and explants *in vitro*, many workers have tried incorporating non-specific absorbents like activated charcoal (AC), antioxidants like polyvinylpyrollidone (PVP) and absorbic acid (AA) in to the culture medium but only met with limited success (Jacquiot 1964, Nitsch and Strain 1969, Constantin *et al.* 1977, Fridborg *et al.* 1978, Weatherhead *et al.* 1979, Bharadwaj and Ramawat 1993). According to George and Sherrington (1984), AC can absorb inhibitory compounds secreted from cultured tissues and thus reduce/avoid the accumulation of phenolic inhibitors. According to Krikorian (1988), the production of brown substances in *in vitro* culture is a reflection of normal response mechanism being incited by the culture process in species capable of producing phenolics in quantity. Recently, Druart and Wulf (1993) reported that addition of AC to the medium reduces browning as well as the sucrose hydrolysis during autoclaving.

The genus *Piper*, is of considerable importance, because it includes cash crops like black pepper (*P. nigrum* L.) and betel leaf (*P. betle* L.), and medicinal plants like long pepper (*P. longum* L. and *P. peepuloides* Roxb.), *P. attenuatum* Ham, tailed pepper (*P. cubeba* L.), and Kava pepper (*P. methysticum* Frost). One of the reasons why in *in vitro* culturing of *Piper* species fails (especially *P. nigrum* L.) is the rapid browning of the medium as well as explants (Jacquiot 1964, Nitsch and Strain 1969). This is mainly due to the presence of phenolics, which were reported to undergo oxidation to produce toxic polyphenols/quinones, which have an inhibitory effect on the growth of explants, resulting in their death (Scalbert *et al.* 1990).

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Abbreviations: AC - activated charcoal; AA - ascorbic acid; MS - Murashige and Skoog's medium; PVP - polyvinylpyrollidone.
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The genus *Piper* contains high concentrations of phenols. Explants when cultured *in vitro* turned brown quickly which results in a considerable loss of cultures. The aim of the present study was to alleviate the damage caused by browning by using activated charcoal (AC) in various concentrations and thereby increase the morphogenetic capability of the various explants of economically important *Piper* species under *in vitro* conditions.

Different explants such as nodal, internodal segments, petiole and leaf were used in the present study for culturing of black pepper (*P. nigrum* L. a cultivar Panniyur-1 and wild species), one local cultivar of betel (*P. betle* L.), long pepper (P. *longum* L.) and *P. attenuatum Ham.*, locally known as 'nayi menasu'. The explants of young plants were cultured on MS basal medium (Murashige and Skoog 1962) with different concentrations of activated charcoal (*Sarabhai Merck Chemicals*, Baroda, India). Since the *in vitro* responses of different *Piper* species have to be studied, to the basal media different concentrations and combinations of growth regulators were added. All media contained 0.8 % agar, pH was adjusted to 5.8 and varying concentrations of AC (50 to 250 mg dm^{-3}) were added. The media were dispensed into tubes and covered with polypropylene caps and autoclaved at 121 °C for 15 min. The explants were washed in running tap water for 30 min, followed by rinsing with a detergent solution (a few drops Tween-20 in water) for 5 - 10 min and then in distilled H_2O. The explants were then surface sterilized with 0.1 % mercuric chloride for 3 - 5 min and washed 3 - 4 times with sterile distilled H_2O to remove all traces of $HgCl_2$ and planted.

Table 1. Degree of browning of the medium or explants at varying concentrations of activated charcoal (AC) in different *Piper* species cultured *in vitro* at the end of the first week of cultivation and percentage of plantlet regeneration.

Piper species	Explants	AC [mg dm^{-3}] Control	50	100	200	250	browning* [%]	regeneration** [%]
P. nigrum	Stem	++++	++++	++++	+++	+++	95.0	-
(wild)	Petiole	++++	++++	++++	+++	++	90.0	-
	leaf	++++	++++	+++	++	+	89.5	-
P. nigrum	Stem	++++	++++	++	+	+	88.8	-
cv.	Petiole	++++	++++	++	+	+	85.0	-
Panniyur-1	leaf	++++	+++	++	+	+	85.0	-
P. attenuatum	Stem	+++	+++	++	-	-	35.0	58.3
	Petiole	+++	++	+	-	-	30.0	-
	leaf	+++	+	+	-	-	27.2	-
P. betle	Stem	+++	++	+	-	-	33.3	66.6
	Petiole	++	+	-	-	-	31.2	-
	leaf	++	+	+	-	-	25.0	-
P. longum	Stem	++	++	+	-	-	15.0	85.3
	Petiole	++	+	+	-	-	12.5	56.2
	leaf	++	+	-	-	-	15.4	73.2

++++ - dark brown, almost black; +++ - brown; ++ - light brown; + - light grey (below the explant); - no colouration; * - percentage of tubes turned brown at 200 mg(AC) dm^{-3} from total tubes inoculated; ** - percentage of plantlets produced after 7 weeks from total cultures inoculated

All the cultures were incubated at 25 ± 3 °C and 16-h photoperiod provided by cool white fluorescent tubes (irradiance 30 - 40 μmol m^{-2} s^{-1}). Subculturing was done once in 20 d. All experiments were carried out in triplicate, each experiment having 20 replications per treatment. The extent of phenolic leaching into medium was assessed on the basis of colour change in medium from creamish brown to almost black. Percentage of browning was calculated by noting the number of tubes turned brown out of total number of tubes inoculated. The percentage of plantlet regeneration was determined by noting the number of tubes produced plantlets out of the total number of cultures.

Leaching of polyphenols into the medium was effectively controlled up to a certain extent in all the explants tested (except for *P. nigrum* wild taxon), by incorporating AC as an adsorbent into the culture medium. Whereas in the case of *P. nigrum* wild stock, the first signs of browning in the explants were visible right after dissection, in other cases the explants turned brown soon after their transfer to the culture media. The same is true in the case of control set in all the taxa studied, but with varying intensities among species, *i.e.*, the highest browning in *P. nigrum* wild taxon and the least in

P. longum.

The addition of AC (100 - 250 mg dm^{-3}) greatly reduced the incidence of browning of the explants of *P. longum*. In *P. betle*, *P. attenuatum* and *P. nigrum* cv. Panniyur-1 it was also found to be helpful in reducing the incidence of browning to a certain extent. However, it was not much helpful in the case of *P. nigrum* wild stock. It was also noticed that the intensity of browning was reduced during subsequent subculturing (Table 1). Here maximum browning was noticed in *P. nigrum* wild stock and least in *P. longum*. The *in vitro* responses of *P. longum, P. betle* and *P. attenuatum* was also studied in the basal medium with complete absence of AC, and in a few instances (less than 20 %) regenerants showed some growth. However, recent findings of Ebert and Taylor (1990), showed that, AC was found to absorb growth regulators, thus limit the utilization of growth regulators in the culture, and therefore the potency of these growth regulators. It is therefore, worthwhile to take up the investigation avoiding the presence of AC in the culture medium, if the morphogenetic responses are encouraging and also try other antioxidants like PVP, AA, *etc.*

The beneficial effects of the incorporation of absorbents such as AC was recognized right after the dissection of explants but not in the later stages of growth, indicating that the mechanism of browning in wounded tissues is different from that in intact plants. The loss of explants/calli was greatest at the beginning of the growth phase. Alleviating these losses by incorporating AC into the culture medium was the major stimulus for the present work. As the propagation of *Piper* species *in vitro* is most difficult to achieve due to phenolics, the greatest attention was paid to overcome this problem. However, further research will be necessary to achieve good multiplication rate and rooting of the explants especially in the case of *P. nigrum* (both wild and cultivated) for germplasm maintenance and crop improvement programme of black pepper.

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