AGRONOMY SCIENCE AND BIOTECHNOLOGY

RESEARCH ARTICLE

Activated charcoal added to tissue culture media increases genotype-dependent biomass production in soybean

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ABSTRACT

Due to its important participation in the agribusiness model worldwide, soybean actively drives national economies in producing countries. However, biotic and abiotic factors caused by pests and climate changes, respectively, can disrupt its productivity and consequently the business market. For this reason, the development of plants more tolerant to these negative environmental elements has been frequently one of the goals of scientific research. In the pipeline to obtain genetically improved plants, tissue culture protocols often represent a bottleneck, since the efficiency at this stage can be genotype-dependent. Therefore, the objective of this work was to evaluate the root regeneration process of two soybean genotypes (BRS 283 and BRS 537) in four different substrates (vermiculite, sand, medium containing activated charcoal and, control – MS medium and glucose). The rooting development was measured by the root's length (cm^2), dry mass (mg), volume (mm^3), surface area (mm^2), and diameter (mm). Results showed that in the activated charcoal medium, for both soybean genotypes, roots grew longer and presented a higher dry mass of roots, and root length when compared to vermiculite and sand substrates. We concluded that the efficiency of tissue culture is genotype-dependent since assayed genotypes presented phenotypical responses significantly different. The supplementation of tissue culture medium with active charcoal improved root growth for both genotypes. Therefore, it is likely that this medium can be also successfully applied to other soybean genotypes, or to other crops with similar tissue culture procedures to promote better rooting and plant establishment in further developmental stages.

Keywords: *Glycine max*, tissue culture, substrate, genotype-dependent, regeneration of explants, rooting regeneration and development.

INTRODUCTION

The soybean *Glycine max* (L.) Merrill is one of the most important crops in the world due to the scale of its production, driving the economy of many producing countries (Ferreira et al., 2021; Treter et al., 2021). In the last Brazilian harvest (2020/2021), the grain production was approximately 136 million tons. The Midwest region is the major grain producer with approximately 62 million tons, followed by the South region with approximately 43 million tons, with Paraná state being the second major producer with approximately 20 million tons (Companhia Nacional de Abastecimento [CONAB], 2021).

Despite these positive numbers, in recent years, production is being impaired by biotic and abiotic factors, which have become more intense and frequent, due to climate change impacts that cause environmental imbalances such as drought, floods, pests and insect infections, diseases, among others, affecting the plant-environment interactions (Ghini, 2006). In this context, the development of plants developed to withstand environmental adversities has become an increasingly frequent target of scientific research. Likewise, tools derived from biotechnology could be a viable and accessible alternative for the development of these genetically improved crops (Barbosa et al., 2012; Fuganti-Pagliarini et al., 2017).

During the development of genetically improved plants, the tissue culture is often one of the most critical stages (Abbasi, Hooshyar, & Bagherieh-Najjar, 2016), since it is genotype-dependent, and it can be strongly affected by the culture medium (Liu, Yang, & Wei 2004; Liu & Wei, 2005). In this context, the main aspect to be considered in the selection of genotypes, besides *A. tumefaciens* infectivity, is their ability to regenerate *in vitro*. Moreover, it is also desirable that the genotypes present excellent agronomic performance (Song et al., 2013; Jia et al., 2015; Marinho et al., 2021).

Soybean genotypes are generally considered recalcitrant for transformation despite many successful examples (Li et al., 2017). In particular, the regeneration of explants can be drastically affected by the substrates used post-transformation. However, no study has shown the relation between different substrates and genotypes within the tissue culture stages of soybean. Therefore, with the objective to improve the soybean tissue culture procedure, we compared the effect of four different substrates/media used for rooting development of two soybean genotypes - BRS 283 and BRS 537. We demonstrated through phenotypic analyses that rooting regeneration and development is genotype-dependent.

Furthermore, we showed that the addition of activated charcoal to MS medium improved the rooting process in both genotypes tested; making it an option to enhance rooting in genetically modified seedlings. Plants need a well-established rooting system to be able to acclimate in a greenhouse, which enables them to complete their life cycle. Our results suggested that charcoal treatment could be applied to different soybean genotypes and to other crops with similar tissue culture protocols, once an improvement in the root system was identified.

MATERIAL AND METHODS

Plant materials and seed sterilization

The soybean genotypes BRS 283 and BRS 537, both from maturity group 6, were recommended by Embrapa's Soybean breeding program team, considering their yield potential and resistance to important pests and diseases. In addition, they have

been used in transformation experiments at the Biotechnology Laboratory at Embrapa Soybean research facility.

Seed sterilization consisted of soaking in 70% ethanol for 1 min, and subsequently in sodium hypochlorite 75% solution for 20 min. After these procedures, the seeds were rinsed three times with sterilized water and transferred to the germination media (GERM) for four days (4d) in a growth chamber set to a temperature of 25-26°C and humidity of 50-70%. Germination media were prepared using B5 Salts (Gamborg) 3.2 g L, B5 vitamins (Vit) 1ml L, MES 0.64 g L, sucrose 30 g L, and Phytaagar 6.5 g L. The media pH was corrected to ~ 5.6-5.7 and subsequently autoclaved. PPM - Plant Preservative Mixture, a relatively new broad-spectrum preservative and biocide used in plant tissue culture, was added to the media (500 µL L) as an alternative to conventional antibiotics and fungicides used in plant tissue culture. Afterward, the germinated seeds were transferred to the four tested growth media.

Substrates for the rooting experiments

The rooting development of each soybean genotype was analyzed in four different growth media (treatments): Control [C = 2.2 g of MS medium (Murashige and Skoog, 1962) + 1 mL of Gamborg's vitamin solution (1000X) + 0.64 g (MES) + 20 g of sucrose)]; Vermiculite (V = 100% vermiculite substrate); Sand (S = 100% sand); Activated charcoal plus control (A = 20 mL of C + 1 g L of activated charcoal). After autoclaved, the media were cooled and poured into plastic cups.

The activated charcoal (A) rooting medium was composed of 20 mL of the control medium mixed with 1 g L of activated charcoal. The vermiculite (V) rooting treatment was composed of plastic cups containing 2.76 g of vermiculite, and the sand (S) treatment was composed of pots containing 44 g of sand each. Every four days, 5 mL of water was added to V and S rooting treatments. The experimental design was performed using completely randomized blocks (4 treatments X 2 cultivars) with nine biological repetitions (n=9). Three replicates were used in the experiments, totaling 72 immature embryos from germinated seeds, 36 per genotype.

Standardization for the root analysis

To standardize the starting growing conditions, the seedlings at the V_1 stage (Fehr & Caviness, 1977) had their primary roots cut off horizontally and, then, transversally to expose the roots to a bigger media/substrate contact surface. The radicular region of the seedlings was immersed on indole-3-butyric acid (IBA) for 1 min before being transferred to each treatment. Roots development was evaluated 22 days later.

Analysis of roots using SAFIRA software

After the end of the treatments, roots were washed, scanned on a printer, and subsequently analyzed using SAFIRA software (Jorge & Silva, 2010), which is a fiber and root analysis system that measures root's length (cm^2), volume (mm^3), surface area (mm^2), and diameter (mm).

Root length and dry mass

After SAFIRA analysis, the seedlings were taken to a drying oven at 65ºC to reach constant weight. Finally, the length of the roots was measured using a ruler and the dry mass by a precision scale.

Statistical analysis

The statistical analysis was performed by SASM-Agri software using ANOVA, followed by the Tukey test ($p \leq 0.05$) (Canteri, Althaus, Virgens Filho, Giglioti, & Godoy, 2001).

RESULTS AND DISCUSSION

A prerequisite for the successful use of genetic transformation methodology is an efficient plant regeneration protocol. For soybean, an important crop worldwide, regeneration of commercial cultivars have been described as ineffective and slow (Raza, Singh, & Bhalla, 2017). Aiming to improve root growth in tissue culture, BRS 283 and BRS 537 soybean cultivars experimented in four different substrates/media post-transformation. Results showed improved growth in length in the roots that were cultivated in MS media containing activated charcoal for both soybean genotypes. BRS 283 and BRS 537 showed 14.4 cm and 13.3 cm long, respectively, in comparison to vermiculite (V) (7.9 cm and 8.5 cm, to BRS 283 and BRS 537 genotypes, respectively) and sand (S) (1.5 cm and 2.8 cm, to BRS 283 and BRS 537 genotypes, respectively) substrates (Figure 1A). A significant difference between the control (C) and the activated charcoal (A) conditions was only observed on BRS 283 genotype ($p \leq 0.05$), a phenotypic response not observed for BRS 537 genotype. This demonstrated that activated charcoal promoted different growth responses in the tested soybean genotypes, behavior also observed by other authors, with different species as well. Dumas and Monteuuis (1995) found that during an *in vitro* test of micropropagation shoots from juveniles and mature *Pinus pinaster*, the addition of activated charcoal in the rooting medium improved the adventitious rooting for the mature explant material (average rooting rates of 78% vs 21%, respectively, in the presence and absence of activated charcoal). Oakes, Desmarais, Powell and Maynard (2016) also observed the same improved results studying root growth in American chestnut sprouts (*C. dentata*) in the presence of activated charcoal. These authors also described that an increase in the concentration of charcoal did have a significant effect on the number of roots ($p < 0.0001$), and length of the longest root ($p \leq 0.0001$).

In this study, the BRS 537 genotype exhibited the highest dry mass in substrates A (15.46 mg) and C (14.00 mg), while in the same treatments, the BRS 283 genotype exhibited dry mass of 11.42 mg (A) and 7.02 mg (C). However, the differences between the dry matters obtained in A and C were significant only for the genotype BRS 537 (1.46 mg, $p<0.05$). When grown in V substrate, both soybean genotypes showed a significantly lower dry mass of roots than the corresponding controls (3.79 mg and 5.5 mg, for BRS 283 and BRS 537, respectively). Finally, no root growth was exhibited in the S substrate, which resulted in a significant difference between these two treatments (C and S) (Figure 1B). The increase in dry mass presented by BRS 537 genotype when compared to its control condition is likely due to the incorporation of the activated charcoal in the medium since no other factor was added. The activated charcoal has a very fine network of pores with a large inner surface area on which many substances can be adsorbed, which may have improved the pH level

balance, in addition to improving nitrogen absorption by the sprouts and inducing rooting (Thomas, 2008; Shekhawat & Manokari, 2016).

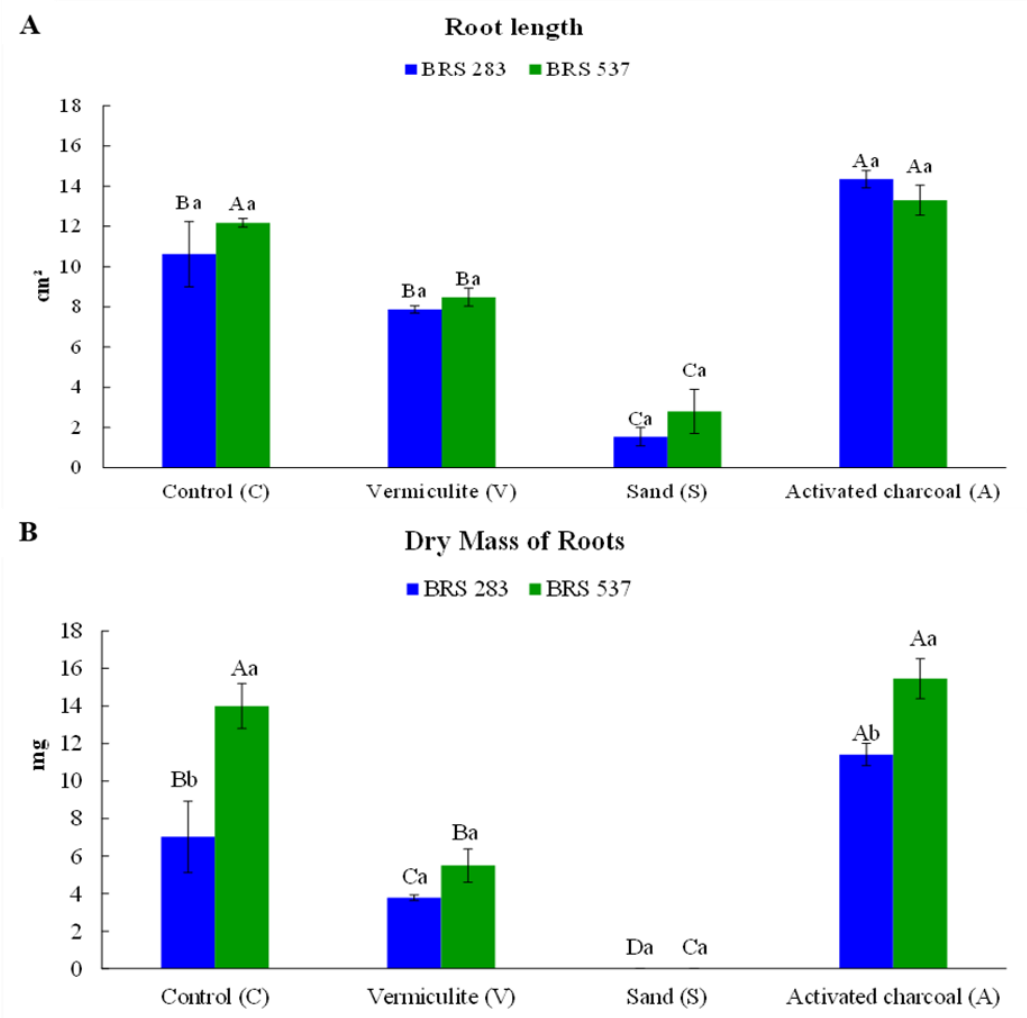

Figure 1. The y-axis displays in A the root length (cm²), and in B the dry root mass (mg). The x-axis displays the treatments: Control (C), Vermiculite (V), Sand (S), and control medium plus activated charcoal (A) as the media for the growth of the soybeans genotypes BRS 283 and BRS 537. Equal capital letters show treatments with no significant mean differences, while equal lowercase letters show genotypes with no significant mean differences (Tukey multiple comparisons test, p≤0.05).

Given the differences observed between both tested soybean genotypes when grown under MS supplemented with activated charcoal, the samples were evaluated using a software to measure and differentiate further characteristics (Figure 2A to 2D). For roots volume, the data showed differences between genotypes. No significant difference was observed for roots volume in BRS 283 genotype, when growing in control (C) and activated charcoal (A); however, BRS 537 genotype showed a higher root volume under control in comparison to the activated charcoal, and higher roots volume than BRS 283 when both were growing in charcoal medium (Figure 2A). The higher root volume exhibited from BRS 537 in charcoal added media may be a result of the cultivar genealogy BRS537 [(BR05-40131 x (ANX-1-55 x BRS 282))] x BRS 284 when compared to BRS 283 (Don Mario 48 x Suprema).

Regarding the roots surface areas, the genotype BRS 537 showed the highest growth in both treatments, C (864.89 mm^2), and A (1722.10 mm^2) media. There was

no statistical difference in surface area between BRS 283 and BRS 537 grown in A, although BRS 537 genotype presented a higher surface area (Figure 2B). Root diameter measurements were also assessed. The BRS 537 genotype grown in activated charcoal presented thicker roots (115.18 mm). Likewise, the genotype BRS 283 presented thicker roots in the same growing conditions (89.07 mm). Under charcoal media, both genotypes developed larger root diameters (Figure 2C). It is important to highlight that the SAFIRA software analyzes fibers and roots by the imagery (BMP, JPEG, or even followed by digital camera or scanners), which facilitates the estimation of volume, surface area and, length by diameter range. The use of activated charcoal in plant tissue culture has been reported to increase shoot formation, plant recovery, and rooting (Thomas, 2008).

In our experiments, we also observed the highest number of secondary roots in both soybean genotypes, BRS 283 and BRS 537, grown in the presence of activated charcoal (A) (Figure 2D). This growth response may result from the adsorptive capability of charcoal, which adheres to toxic substances such as phenols, and to the excess of growth regulators that can accumulate in the culture medium, impairing root development. Also, charcoal can mimic the soil as it reduces the incidence of light in the growth zone root system (Bonga, 1985; Mantovani, Franco, & Vestena, 2001).

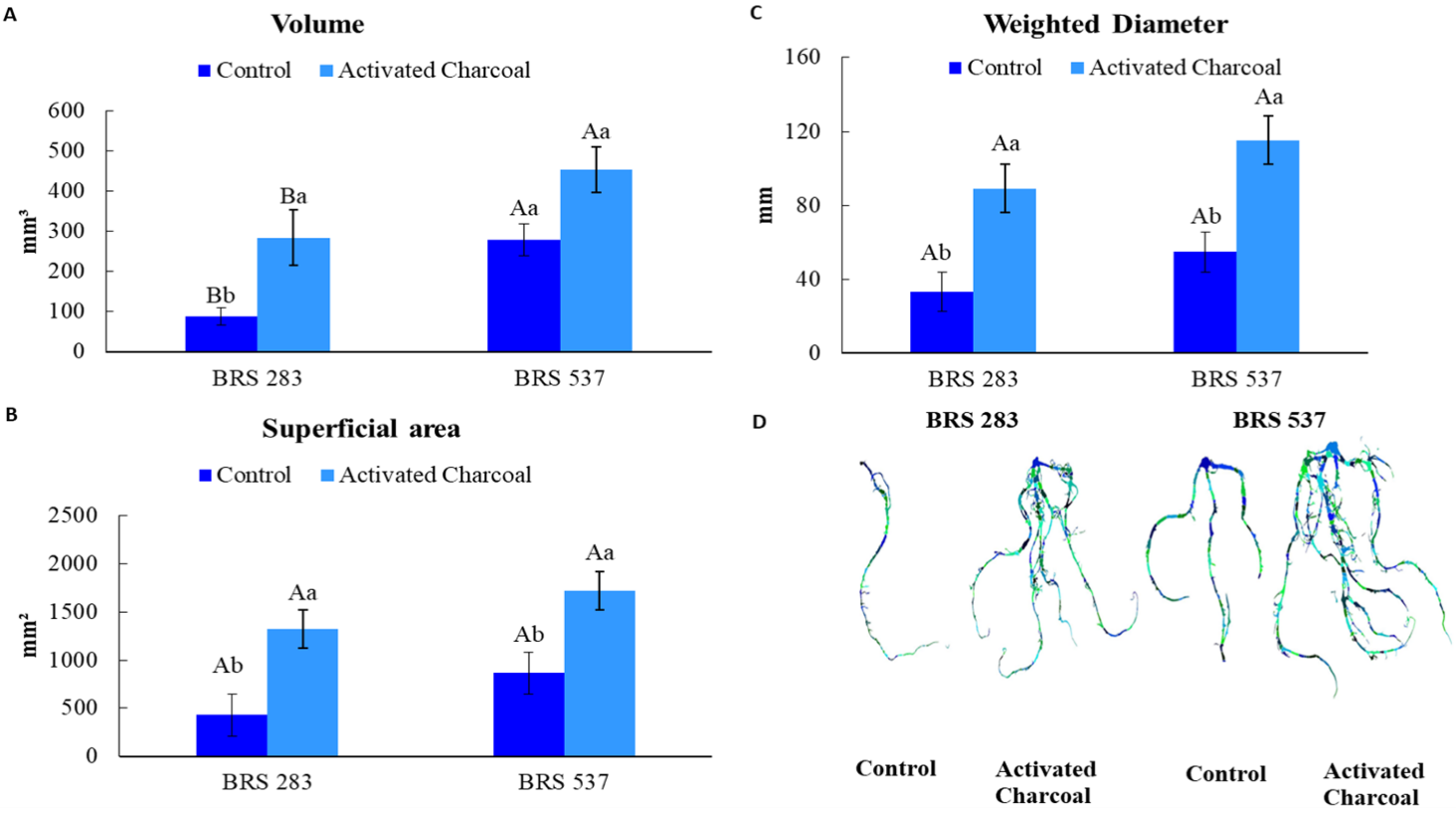

Figure 2. In A, data from root's volume (mm³); in B, root's surface area (mm²); in C, weighted diameter (mm), and in D, visual comparison generated by the SAFIRA software comparing the control condition (C) and MS with activated charcoal added (A), for the two soybean genotypes analyzed, BRS 283 e BRS 537. Capital letters compare genotypes and lower letters compare treatments for the same genotype (Tukey multiple comparisons test, p≤0.05).

Phenotypically, the rooting process under the activated charcoal condition showed greater improvement for the BRS 537 genotype (Figure 3B) when it was

compared with the BRS 283 genotype (Figure 3A). In general, for both genotypes, control substrate (C) and the activated charcoal (A) showed the best root's development. Similar root growth patterns in medium containing activated charcoal were also identified in Macadamia (*Macadamia tetraphylla*) (Mulwa & Bhalla, 2006), mulberry (*Morus alba*) (Agarwal & Kanwar, 2007), banana (*Musa acuminata*) (Xiao et al. 2007), salvia (*Salvia africana-lutea*) (Makunga & staden, 2008) and in soybean (*Glycine max*) developed by embryo differentiation (Klink et al. 2008). All these data suggested that charcoal could lead to an increase in sprouts and, consequently, root elongation in length, of both soybean genotypes, not essentially having an effect on root diameter and volume. In general, BRS 537 genotype responded better to charcoal addition in the growing medium when compared to BRS 283 genotype, as all assayed data showed.

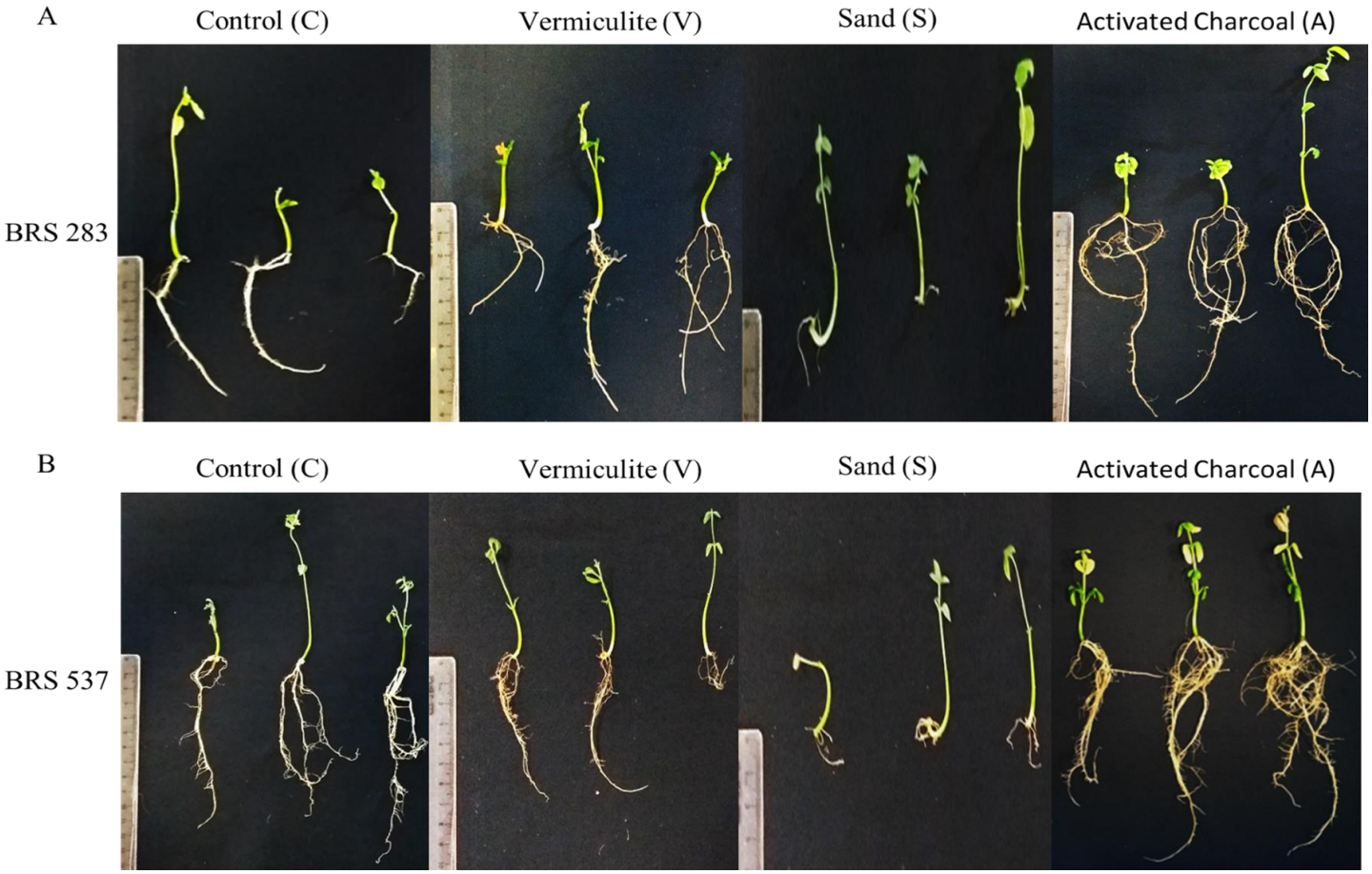

Figure 3. Images show root growth for the two soybean genotypes, BRS 283 (A) e BRS 537 (B), in 04 different treatments. (C) control medium, vermiculite (V) substrate; sand (S) substrate, and MS medium plus activated charcoal (A).

The summary of characteristics that showed statistical difference comparing control conditions with activated charcoal added medium is described in Figure 4. It's important to highlight that having a cultivar, such as BRS 537, showing a good root development as a control in the tissue culture stages provide the researchers a standard model for comparison with tested cultivar as well as a control for the media with added charcoal. This protocol could also be applied in screenings with many soybean candidate cultivars, ensuring that cultivars with good root development were chosen for a genetic transformation process.

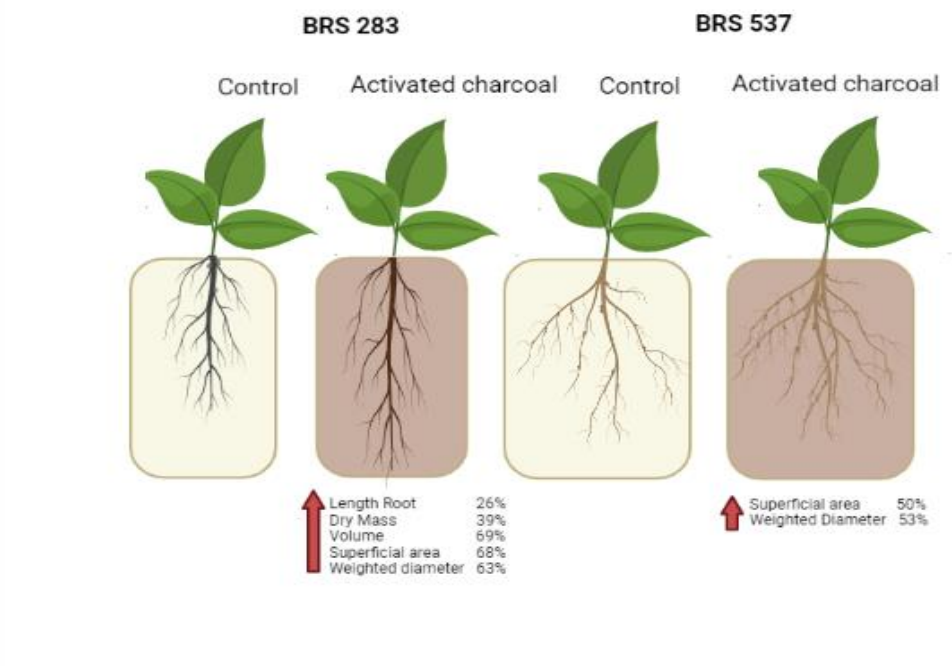

Figure 4. Characteristics that showed statistical difference comparing control conditions (MS medium, Gamborg's vitamin solution and sucrose) with activated charcoal treatment (C + 1g L activated charcoal) for both soybean genotypes assayed, BRS 283 and BRS 537.

CONCLUSIONS

Results pointed out that rooting growth efficiency in soybean tissue culture was genotype-dependent, for the two cultivars tested.

The great contribution of the work is in the characterization of two soybean genotypes development in different culture media, which led to the conclusion that there is a subtract x genotype interaction and to the conclusion that the medium with activated charcoal offered the best chances of success in tissue culture for both genotypes, considering all data assayed.

Medium containing activated charcoal induced better root growth in both genotypes, BRS 283 and BRS 537, as identified in the length and dry mass of roots data. However, BRS 537 showed higher roots volume, superficial area, and diameter.

Regardless of the soybean cultivar, rooting development at vermiculite and sand substrates showed poorer growth performance.

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