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In vitro growth optimization and essential oil composition of basil cultivars

Abstract – The objective of this work was to evaluate the effect of the use of the Murashige & Skook (MS) and Linsmaer & Skoog (LS) media and of filter caps on culture flasks on the morphological and physiological characteristics of basil cultivars in vitro, as well as to determine the composition of the essential oil of plants acclimatized in pots. For the evaluation of the cultivation media, cultivars Anise, Cinnamon, Grecco a Palla, and Italian Large Leaf were used. The Anise, Cinnamon, Italian Large Leaf, and Maria Bonita cultivars were used for the evaluation of the use of filter caps. The composition of the essential oils of cultivars Anise, Cinnamon, and Italian Large Leaf was also evaluated. The basil seedlings showed a higher aerial-part dry mass and a lower hyperhydricity rate when grown in the MS medium. The flasks without filter caps produced seedlings with lower contamination rates. When acclimatized in pots, cultivars Limoncino and Anise presented the tallest plants with the largest crown diameters. In the composition of essential oils, methyl cinnamate and linalool stood out in cultivar Cinnamon, methyl chavicol in Anise, and linalool in Italian Large Leaf. In the in vitro cultivation, the evaluated cultivars present a higher aerial-part production in the MS medium and a lower contamination without the use of filters, whereas, in the pots, the composition of essential oils varies according to the cultivar.

Index terms: *Ocimum basilicum*, culture media, filter caps, micropropagation.

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Optimização do crescimento in vitro e composição de óleos essenciais de cultivares de manjericão

Resumo – O objetivo deste trabalho foi avaliar o efeito do uso dos meios Murashige & Skook (MS) e Linsmaer & Skoog (LS) e de tampas com filtros nos frascos de cultivo sobre as características morfológicas e fisiológicas de cultivares de manjericão in vitro, bem como determinar a composição do óleo essencial de plantas aclimatizadas em vasos. Para a avaliação dos meios de cultivo, foram utilizadas as cultivares Anise, Cinnamon, Grecco a Palla e Italian Large Leaf. As cultivares Anise, Cinnamon, Italian Large Leaf e Maria Bonita foram usadas para a avaliação do uso de tampas com filtros. Avaliou-se, também, a composição dos óleos essenciais das cultivares Anise, Cinnamon e Italian Large Leaf. As plântulas de manjericão apresentaram maior massa seca da parte aérea e menor taxa de hiperidricidade quando cultivadas em meio MS. Os frascos com tampas sem filtro produziram plântulas com menores índices de contaminação. Quando aclimatizadas em vasos, as cultivares Limoncino e Anise apresentaram as plantas mais altas e com os maiores diâmetros de copa. Na composição de óleos essenciais, destacaram-se metil cinamato e linalol na cultivar Cinnamon, metil chavicol na Anise e linalol na Italian Large Leaf. No cultivo in vitro, as cultivares avaliadas apresentam maior produção de parte aérea em meio MS e menor contaminação sem uso de filtros, enquanto,

em vasos, a composição de óleos essenciais varia de acordo com a cultivar.

Termos para indexação: *Ocimum basilicum*, meios de cultura, tampas com filtro, micropropagação.

Introduction

Basil (*Ocimum basilicum* L., Lamiaceae) is a medicinal, culinary, and aromatic plant that is native to Africa and, possibly, Asia (Amaral-Baroli et al., 2016; Welz et al., 2020). Several *Ocimum* species have attracted research attention because they contain essential oils with more than 20 components, including methyl chavicol, methyl cinnamate, eugenol, citral, linalool, thymol, estragole, geraniol, calarene, camphor, and tannins (Silva et al., 2017), which can even be used to control agricultural pests (Silva et al., 2017; Singh et al., 2021). This growing interest in medicinal plants has led to an increase in the demand for the identification of their by-products and active ingredients, aiming for their better commercialization and conservation (Srivastava et al., 2018), which also requires studies on the optimal concentrations, quantities, and qualities of their essential oils.

To optimize the propagation of aromatic species while maintaining their desirable traits, plant tissue culture is preferred because it allows of mass (micro) propagation without the significant heterogeneity that results from propagation by seeds, facilitating the inclusion of the species in germplasm banks (Bandinelli et al., 2013).

In addition to the type of propagation, the type of culture media is also important in in vitro basil cultivation. Among the used media, that of Linsmaier & Skoog – LS (Linsmaier & Skoog, 1965) differs from that of Murashige & Skoog – MS (Murashige & Skoog, 1962) because it does not contain nicotinic acid, pyridoxine, and the amino acid glycine, but contains more of the vitamin thiamine. According to Salisbury & Ross (2012), not all plants are able to synthesize their own vitamins, so the incorporation of these substances into the environment is a viable option.

Another factor that affects in vitro basil cultivation is the type of seal used on the tissue culture flasks since the quality of the microenvironment inside the flasks is influenced by gas exchange with the external environment (Walli et al., 2019; Silva et al., 2022). Therefore, the types of flask caps and the type and quantity of media determine the microenvironment within the flask and, consequently, the variability in the behavior of cultures and their products. However, up to date, few studies have tested cultivars with the aim of optimizing in vitro growth and essential oil composition.

The objective of this work was to evaluate the effect of the use of the MS and LS media and of filter caps on culture flasks on the morphological and physiological characteristics of basil cultivars in vitro, as well as to determine the composition of the essential oil of plants acclimatized in pots.

Materials and Methods

Three experiments were carried out at the Biotechnology Laboratory of Universidade Federal de Uberlândia, located in the state of Minas Gerais, Brazil. In the first, the effect of the MS and LS culture media on four basil cultivars was evaluated; in the second, the use of filter caps on flasks was tested using four basil cultivars; and, in the third, the composition of the oil extracted from three cultivars acclimatized in pots was determined.

The used basil cultivars were: Anise, Cinnamon, Grecco a Palla, and Italian Large Leaf in the first experiment; Anise, Cinnamon, Italian Large Leaf, and Maria Bonita in the second; and Anise, Cinnamon, Italian Large Leaf, and Limoncino in the third, with only the first three being used for oil extraction. Seeds from cultivars Anise, Cinnamon, Grecco a Palla and Italian Large Leaf were obtained from Richters Herbs (Goodwood, Ontario, Canada) and used as an explant source. The Limoncino and Maria Bonita cultivars were provided by the Breeding Program of Aromatic Plants of Universidade Federal de Sergipe (São Cristovão, SE, Brazil).

The first experiment was set up in a randomized complete block design, in a 4×2 factorial arrangement (basil cultivars Anise, Cinnamon, Grecco a Palla, and Italian Large Leaf and MS and LS culture media), with five replicates. Each experimental plot consisted of ten flasks containing five seeds.

All seeds were disinfected in 70% alcohol for 1 min and in 30% sodium hypochlorite solution for 20 min. Afterwards, the seeds were washed with distilled water in a laminar flow chamber and then autoclaved.

The MS and LS culture media were prepared in 200 mL flasks at 50% concentrations using 1.8 g L^{-1} Phytagel (Sigma-Aldrich, São Paulo, SP, Brazil), 3.0% saccharose, and pH adjusted to 5.7, being autoclaved at 121°C and 1.2 atm for 20 min. Seeds of each cultivar were inoculated in flasks containing sterilized culture media, which were placed in a growth chamber for 60 days under a 16-hour photoperiod, 25±2°C temperature, and 25 μmol $m^{-2}s^{-1}$ light intensity from white fluorescent lamps. After 60 days of explant inoculation, the following characteristics were evaluated: hyperhydricity rate based on the scale from Trento (2017), leaf number, shoot number, shoot length (cm), root length (cm), and shoot dry mass (g). Shoot dry mass was determined after drying in an oven, at 65°C, for 72 hours, whereas shoot length was measured using a ruler.

The second experiment was conducted in a randomized complete block design in a 3×4 factorial arrangement (caps with one, two, or no filters and cultivars Anise, Cinnamon, Italian Large Leaf, and Maria Bonita), with five replicates, using only the MS culture media due to its better performance in the previous trial. Each experimental plot consisted of five flasks that were sealed with polypropylene caps with one, two, or no filters and that contained five explants each. The caps had one or two 5.0 mm holes, covered with white microspore tissue. After 60 days of inoculation, the following characteristics were evaluated: contamination rates in developed seedlings (%), considered every flask that showed any sign of microorganism proliferation; the soil plant analysis development (SPAD) index, using the SPAD-502 index chlorophyll meter (Konica Minolta, Inc., Tokyo, Japan); and leaf number, shoot number, shoot length, root length, and shoot dry mass, as in the first experiment.

In the third experiment, cultivars Anise, Cinnamon, Italian Large Leaf, Maria Bonita, and Limoncino were acclimatized in pots. The seeds were inoculated in MS medium, without cap filters, and were kept in a growth room for 60 days. From a seedling, two or three nodal segments were produced and inoculated in the previously described MS medium, with the addition of 0.5 mg L^{-1} 6-benzilaminopurina based on Trento (2017). After 60 days, the seedlings from the nodal segments were evaluated for hyperhydricity using the scale from Trento (2017). The seedlings considered physiologically normal were transplanted to expanded polystyrene trays (128 cells) and maintained inside the growth chamber. At 30 days in the trays, seedling trios were transplanted into pots (11.8 cm diameter × 8.6 cm diameter × 9.0 cm height) containing a substrate made up of equal parts of cow manure, Plantmax substrate (Paulínia, SP, Brazil), and sand to improve plant growth, increase vegetative mass, and enable oil extraction. At this stage, the plants were kept under a 4.5 m high structure with open sides to protect them from adverse weather conditions.

The plants in the pots were fertilized every two weeks with the Hoagland (Hoagland et al., 1938) nutrient solution until field capacity (100 mL). Irrigation was carried out daily, also until reaching field capacity. Weeds were removed manually, and pests and diseases were monitored daily.

At 30 days after transplanting, non-destructive evaluations started to be carried out in order to measure shoot length (base to apex) and green color intensity of the third or fourth mature and fully-expanded leaf from the apex, using the SPAD-502 portable chlorophyll meter (Konica Minolta, Inc., Tokyo, Japan).

The crown diameter (cm) and shoot length of the plants were measured using a caliper and a ruler, respectively. At the end of their development cycle (60 days), the entire plants were cut 15 cm above the soil and weighed on an analytical balance to obtain shoot fresh mass (g). To obtain water rate (%) and shoot dry mass, the samples of each plant were placed in paper bags, dried in an oven, at 40°C, for 72 hours (Santana et al., 2018), until reaching a constant mass, and, then, weighed again. Leaf area was determined on the third fully-expanded leaf from the apex using the Easy Leaf Area software (Easlon & Bloom, 2014).

Of the plants acclimatized in pots, those of cultivars Anise, Cinnamon, and Italian Large Leaf were used for essential oil extraction from dry leaf samples via hydrodistillation using a modified Clevenger apparatus (Glassco Laboratory Equipments Pvt. Ltd., Ambala Cantt, Haryana, India). Each extraction was performed in triplicate for 140 min using 30 g dry leaves and 1.5 L distilled water (Ehlert et al., 2006). The essential oils were stored in amber bottles, at -20°C, until their analysis. To determine the oil content (%) in each sample, the volume of essential oil extracted from each sample was divided by the dry mass of each sample.

The analysis of the chemical composition of the essential oils was performed using the GCMS-QP2010 Ultra gas chromatograph mass spectrometer, equipped with the AOC-20i autosampler (Shimadzu Corporation, Kyoto, Japan). Separations were carried out using the 30 m × 0.25 mm i.d. Rtx-5 MS fused silica capillary column (Restek, Centre County, PA, USA), coated with 5%-diphenyl-95%-dimethyl polysiloxane phase, with a 0.25 mm film thickness, at a constant helium (99.999%) flow rate of 1.2 mL min^{-1}. An injection volume of 0.5 µL (5.0 mg mL^{-1}) was used, with a 1:10 split ratio. Oven temperature was programmed at 50°C for 1.5 min isothermal holding, with an increase of 4°C per minute up to 200°C and, then, of 10°C per minute up to 250°C, ending with a 5 min isothermal holding at 250°C.

The mass spectrometry and flame ionization detection (FID) data were simultaneously acquired by the Detector Splitting System (Shimadzu Europe GmbH, Duisburg, Germany), with a mass spectrometry:FID split flow ratio of 4:1. A 0.62 m × 0.15 mm i.d. and a 0.74 m × 0.22 mm i.d. restrictor tube (capillary column) was used to connect the splitter to the mass spectrometry and FID detector, respectively. The mass spectrometry data (total ion chromatogram) were obtained in a full scan mode (m/z of 40–350) at a scan rate of 0.3 s, using electron ionization with an electron energy of 70 eV.

For FID, the injector temperature was 250°C, the ion-source temperature was 200°C, and the gas supplies were hydrogen, air, and helium at the flow rates of 30, 300, and 30 mL min^{-1}, respectively. The quantification of each constituent was estimated by FID peak area normalization (%). The compound concentrations were calculated from the gas chromatography (GC) peak areas and then arranged in the order of the GC elution.

For mass spectrometry, the retention index was obtained by injecting a C7–C30 linear hydrocarbon mixture under those same conditions. The individual components were identified via computerized matching of the acquired mass spectra with those stored in the NIST21, NIST107, and WILEY8 mass spectral library of the GC/mass spectrometry data system, followed by a comparison of the retention index and mass spectrometry values with those reported in the literature (Adams, 2017).

The data obtained in all experiments were subjected to the analysis of variance, and the assumptions of normality of residues, homogeneity of variances, and additivity of blocks were tested and met by the Shapiro-Wilk, Levene, and Tukey tests, at $\alpha = 0.01$, respectively, using the RStudio software (R Core Team, 2020).

Results and Discussion

There was a significant interaction between culture medium and cultivars for leaf number and shoot length. Cultivar Anise showed a 94% increase in leaf number in the MS culture medium, whereas, in the LS medium, cultivar Grecco a Palla produced two to three times more leaves than the others. Regarding shoot length, that of cultivar Cinnamon was 31% higher in the MS media, being superior to those of all cultivars, two times that of Grecco a Palla, and 50% greater than that of Italian Large Leaf. In the LS medium, the plants of cultivars Cinnamon and Grecco a Palla were twice as tall as those of Anise (Figure 1).

Therefore, the used medium influences the performance of basil cultivars, probably due to the impact of its composition on plant metabolism. The MS medium has a higher concentration of nicotinic acid, pyridoxine, and glycine, which had a positive impact on the Anise and Cinnamon cultivars. In the literature, it has been shown that nicotinic acid (niacin or vitamin B) is an alkaloid involved in photosynthesis and that the vitamin pyridoxine participates in energy production and in the synthesis of proteins, lipids, and acetylcholine (Linnell, 2000), while glycine is an amino acid precursor of chlorophyll synthesis that also acts in plant defense mechanisms (Di Gioacchino et al., 2020).

The concentration of these elements in the evaluated media also caused different responses in the cultivars regarding the hyperhydricity rate of the plants (Figure 1), which was 16% in the LS medium for Cinnamon, higher than that of 12% in the MS medium for Italian Large Leaf. However, cultivar Italian Large Leaf had the shortest root length, which was 59 and 79% shorter than those of Anise and Cinnamon, respectively, whereas Grecco a Palla and Italian Large Leaf presented a shoot number twice as high as that of Anise. Overall, hyperhydricity was 30% lower in the MS medium, but shoot mass was three times higher than in the LS medium, which could be associated with the nutritional enrichment provided by the first

medium, responsible for improving ionic strength and better meeting the nutritional requirements of the cultivars (Neponuceno et al., 2014). Since it resulted in a better general plant performance, the MS medium was used in the following sequential experiments.

As to the influence of filter use, there was no interaction between cultivars and filters for percentage of contamination (Figure 2). Without the filters, contamination was 30 and 44% lower than when one and two cap filters were used, respectively. However, for leaf number, SPAD index, shoot length, and root length, there was an interaction between cultivars and filter use. Italian Large Leaf produced more leaves than the other cultivars when kept in the flasks without filters, with 55 and 69% more leaves than Maria Bonita and Cinnamon, respectively, and twice as many as Anise. However, this was not the case for Italian Large Leaf in the flasks with one or two filters, probably because, compared with the other cultivars, it requires more moisture, which is better retained in flasks without filters. In addition, flasks with cap filters facilitate gas exchange, leading to greater water losses. Similarly, Matuszkiewicz et al. (2019) found that two-week-old seedlings of *Arabidopsis thaliana* (L.) Heynh. showed stress symptoms in flasks with filters, which could be related to the sensitivity of some individuals, such as those of the *Ocimum* genus, to moisture levels or even to filters that allow of excessive water losses. Conversely, cultivar Maria Bonita produced 57% more leaves in the flasks with two filters than in those with one filter, producing 50% more leaves than Anise and Italian Large Leaf and twice as many as Cinnamon, meaning it reacts better to an environment with gas exchange.

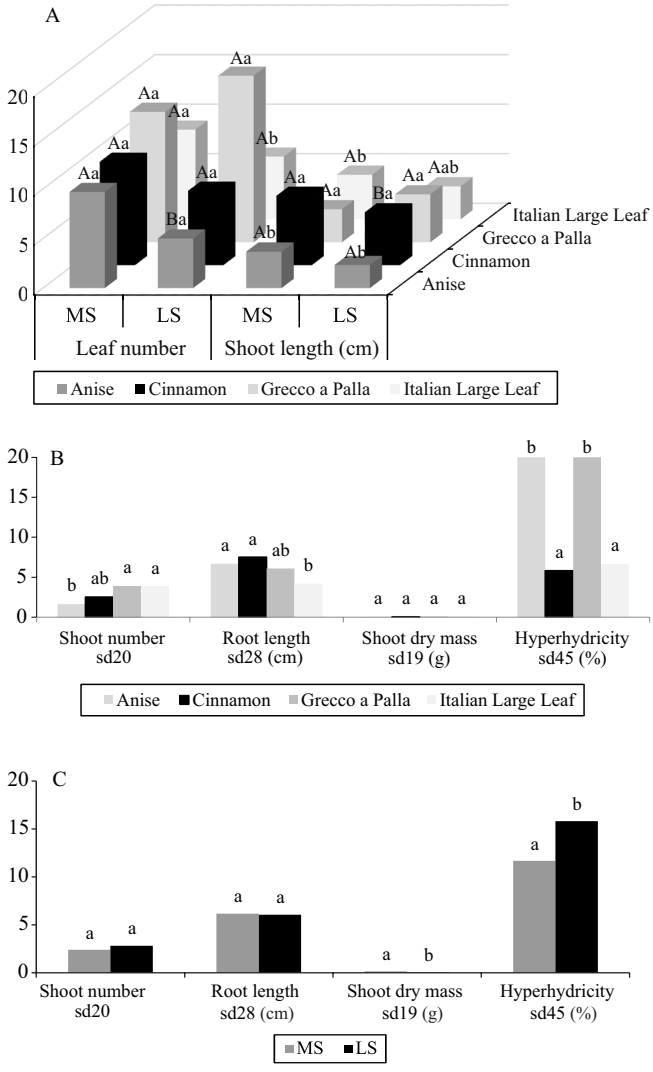

Figure 1. Leaf number and shoot length (A) and shoot number, root length, shoot dry mass, and hyperhydricity rate of the Anise, Cinnamon, Greco a Pala, and Italian Large Leaf basil (*Ocimum basilicum*) cultivars cultivated in vitro in the Murashige & Skook (MS) and Linsmaer & Skoog (LS) culture media. Columns followed by equal letters mean that averages do not differ by the Tukey test, at 5% probability. Sd, standard deviation.

These results are an indicative that the abiotic factors involved in the ambience of in vitro cultivation cause many impacts on the growth and development of seedlings (Walli et al., 2019). A high relative humidity, for example, alters seedling metabolism, which can lead to stress and hormone accumulation (Nguyen et al., 2020). Therefore, information about plant responses to different abiotic conditions can lead to better in vitro performance and ex vitro adaptability (Pepe et al., 2022). In this line, although the use of filters is related to better in vitro development conditions, to the optimization of micropropagation, and to the greater survival of acclimatized seedlings (Silva et al., 2022), the same condition is not always favorable to all genotypes, as observed in the present study, reinforcing the importance of studying the impact of the microenvironment on different cultivars.

Still regarding the effect of filter use, the values of the SPAD index varied. Without filters, the values obtained for cultivar Anise were twice those of Cinnamon and 90% higher than those of Maria Bonita

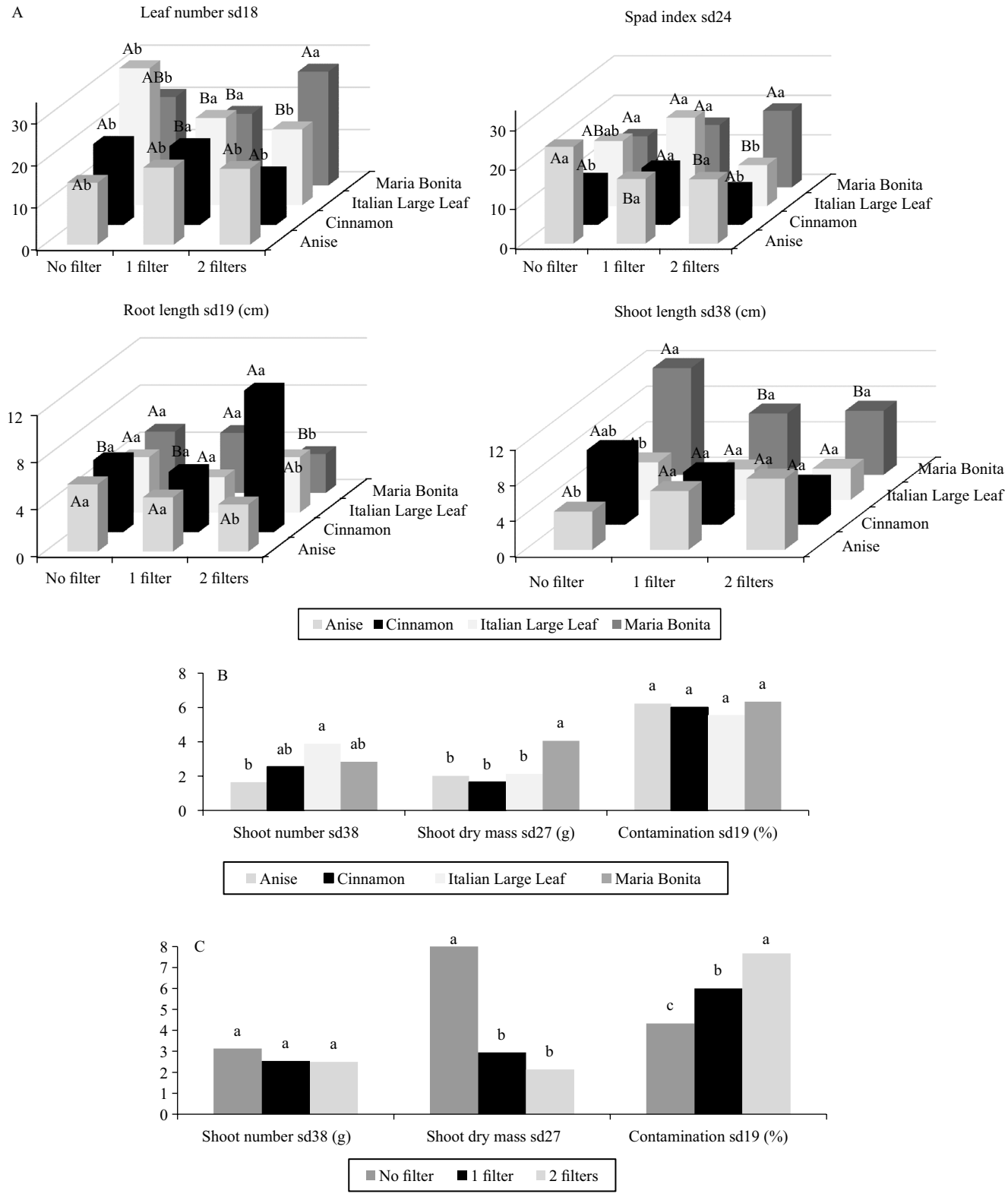

Figure 2. Leaf number, SPAD index, root length, and shoot length (A) and shoot number, shoot dry mass, and rate of contamination (B and C) of the Anise, Cinnamon, Italian Large Leaf, and Maria Bonita basil (*Ocimum basilicum*) cultivars cultivated in vitro in flasks with caps with one, two, and no filters. Columns followed by equal letters mean that averages do not differ by the Tukey test, at 5% probability. Sd, standard deviation.

(Figure 2). In the flasks with one and two filters, respectively, the SPAD index values of the Anise cultivar were 49 and 50% higher than in the flasks without filters. In the presence of two filters, cultivar Maria Bonita presented a SPAD index 87% higher than that of Italian Large Leaf and two times greater than that of Cinnamon. In the case of the Italian Large Leaf cultivar, the SPAD index was two times greater in the flasks with one filter than in those with two filters.

As to the height of seedlings, those of cultivar Maria Bonita were 91.3 and 83% taller in the flasks without filters than in those with one and two filters, respectively, besides being three times taller than the seedlings of Anise and Italian Large Leaf under the same condition (Figure 2). Considering root development, significant differences were only observed with the use of two filters, with cultivar Cinnamon standing out with a root length of 3.3, which was 2.8 higher than those of Anise and Italian Large Leaf and 4.0 times higher than that of Maria Bonita.

For shoot number and dry mass, there was no significant interaction between cultivars and filter use, meaning these characteristics were evaluated separately. Italian Large Leaf had 2.4 more shoots than Anise, whereas Maria Bonita showed a shoot dry mass two times greater than that of the other cultivars. Moreover, in the flasks without filters, shoot dry mass was 2.9 and 4.0 times greater than in those with one and two filters, respectively.

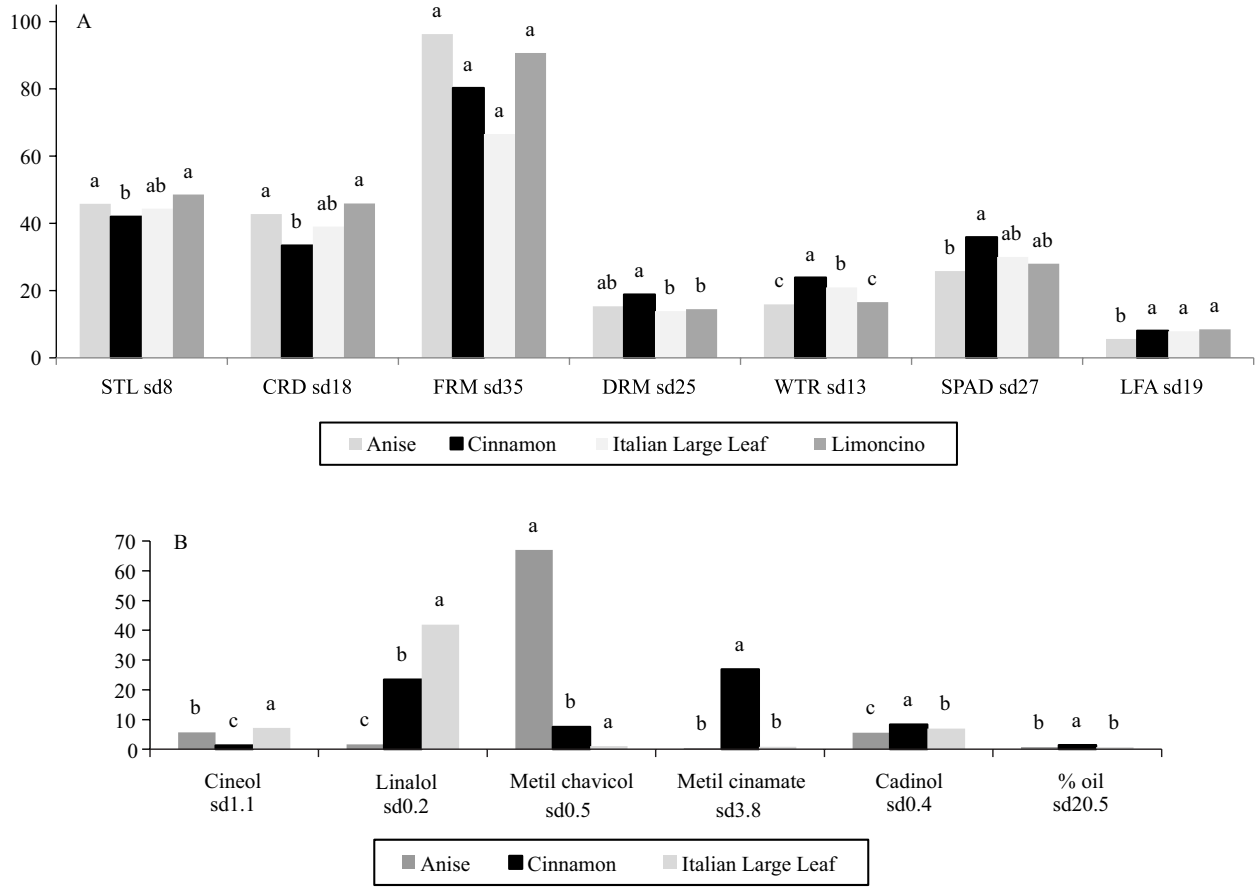

Figure 3. Shoot length (STL, cm), crown diameter (CRD, cm), shoot fresh mass (FRM, g), shoot dry mass (DRM, g), water rate (WTR, %), SPAD index, and leaf area (LFA, cm) of the Anise, Cinnamon, Italian Large Leaf, and Limoncino basil (*Ocimum basilicum*) cultivars acclimatized in pots (A), as well as the percentages of the major components in the essential oil extracted from cultivars Anise, Cinnamon, and Italian Large Leaf (B). Columns followed by equal letters mean that averages do not differ by the Tukey test, at 5% probability. Sd, standard deviation.

When grown in pots, compared with Cinnamon, cultivars Limoncino and Anise stood out with a 16 and 9% longer shoot length and a 37 and 28% greater crown diameter, respectively (Figure 3). The Cinnamon cultivar, however, had 35 and 30% more shoot dry mass than Italian Large Leaf and Limoncino, respectively. As for water rate, cultivar Cinnamon showed values 14, 44, and 50% higher than those of Italian Large Leaf, Limoncino, and Anise, respectively. Regarding the SPAD index, the values obtained for the Cinnamon cultivar were 40% higher than those of Anise, whose leaf area was 32, 30, and 35% smaller than those of Cinnamon, Italian Large Leaf, and Limoncino, respectively.

Regarding the composition of the essential oils of the cultivars, that of Anise consisted mostly of 66.98% methyl chavicol, while that of Cinnamon contained 26.95% methyl cinnamate and 23.44% linalool (Figure 3). The most important alcohols in the Italian Large Leaf cultivar were 1.8-cineole and linalool. Evaluating basil in an in vitro test followed by acclimatization, Amaral-Baroli et al. (2016) found that the main oil compounds from acclimatized seedlings were linalool, 1,8-cineole, and camphor. For 'Sweet Thai' basil, Manan et al. (2016) studied the protocols for micropropagation from shoot tips and the essential oil content at different plant developmental stages, observing that the oil of in vitro seedlings contained 94% methyl chavicol, which was only 60% in the oil of in vitro acclimatized seedlings. Therefore, one of the advantages of in vitro cultivation for the market is the possibility of obtaining especially high-value phytochemicals such as essential oils (Chandran et al., 2020).

It should be noted, however, that, although cultivars contain very similar genetic material, some differences in the environment can modify their phenotypes (Welz et al., 2020). These authors added that the morphological and physiological responses observed in the growth of basil plants are the product of the interaction between culture medium, genotype, and growth conditions as light, photoperiod, and temperature. In the present work, the evaluated basil plants developed better in a certain culture medium and microenvironment, showing that, to optimize in vitro, ex vitro, and phytochemical-related performance, it is necessary to study abiotic factors and cultivars to contribute for the development of a general micropropagation protocol for the species.

Conclusions

1. Basil (*Ocimum basilicum*) seedlings show a higher shoot dry mass and a lower hyperhydricity rate when cultivated in the Murashige & Skook culture medium.

2. In vitro flasks without filter caps produce seedlings with lower contamination rates.

3. The major compounds of the essential oils from basil plants acclimatized in pots are methyl cinnamate and linalool for cultivar Cinnamon, methyl chavicol for Anise, and linalool for Italian Large Leaf.

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