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Sucrose and hyperhydricity in the micropropagation of garlic seeds *in vitro*[1]

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ABSTRACT - In agricultural crops that produce bulbs, the most important stage when the crop is generated via micropropagation is bulb production, as it involves a sensitive transition from the sprout to the bulb production phase. Since garlic is mainly propagated vegetatively from seeds produced *in vitro*, it is important to understand and control the factors that are involved in the process of bulb formation. The aim of this study was to examine hyperhydricity and biometry and their correlation following the transition from shoot to bulb in the *in vitro* seedlings of two noble garlic cultivars, 'Ito' and 'Quiteria', in MS culture medium with sucrose at concentrations of 0%, 2%, 4%, 6%, 8% and 10%. It was found that adding sucrose to the culture medium at concentrations of 8% and 10% during bulb formation resulted in better productive characteristics for the cultivars, with a high percentage of purple (Grade 1) and dark brown (Grade 2) bulbs, which according to the scale, corresponds to low hyperhydricity. The 'Ito' cultivar produced greater percentage bulb formation and a greater quantity of bulbs per plant than the 'Quiteria' cultivar. There was also a strong negative correlation between the vegetative characteristics of fresh root and shoot weight with the reproductive characteristics of number of bulbs per plant and fresh bulb weight, showing that during bulb production the plant directs efforts towards bulb formation to the detriment of vegetative growth.

Keywords: *Allium sativum*. Biometrics. Cultivars. Tissue culture.

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INTRODUCTION

In addition to its use as a condiment, garlic (*Allium sativum* L.) is consumed worldwide for its high medicinal and nutritional value and its biological activity (Nassur *et al*., 2020). Only 17 countries are responsible for approximately 95% of the world's garlic production, with China producing 76% of this total. Brazil ranks 13th, and therefore needs to import part of its consumption (FAOSTAT, 2021).

In Brazil, the most widely planted noble garlic cultivars include 'Ito' and 'Quiteria'. These cultivars stand out for their agronomic and commercial characteristics. The 'Ito' variety is considered extremely productive, with large bulbs and a high level of market acceptance, while'Quiteria' is popular in colder climates, and has large and well-distributed bulblets, that meet the demands of the consumer market.

The species is mainly produced vegetatively, with tissue culture used as a tool to assist in the mass production of homogeneous propagating material of high phytosanitary quality. Among the important factors in bulb formation and the *in vitro* cultivation of garlic are the concentration of soluble carbohydrates and sucrose in the culture medium and its relationship with the hyperhydricity of the *in vitro* tissue (Liu *et al*., 2017; Taha, 2017).

Hyperhydricity is a common morphological, anatomical and physiological disorder when cultivating plants *in vitro* that seriously affects the regeneration and micropropagation of plants such as garlic, and results in losses (Liu *et al*., 2017). In this species, such effects are increased by high concentrations of cytokinin and reduced by increasing concentrations of sucrose and gelling agent, better ventilation and greater light intensity (Liu *et al*., 2017; Oksana; Bedarev, 2022).

In plant tissue culture, sucrose acts as a source of fuel to maintain photomixotrophic metabolism and ensure optimal development. It also helps to maintain osmotic potential and water conservation in the cells (Gao *et al*., 2024; Panis; Nagel; Van den Houwe, 2020). In addition, earlier studies have shown that seedlings grown in a culture medium do not fix enough carbon dioxide to sustain growth in the absence of sucrose; this is mainly due to the limited concentration of carbon dioxide in the culture flask (Aluko *et al*., 2021).

The sucrose present in the culture medium generally undergoes partial or complete hydrolysis following the autoclaving process, which results in glucose and fructose as monosaccharide products, and facilitates absorption by the plant tissue (Aluko *et al*., 2021). However, relatively few studies have been dedicated to the effects of these monosaccharides on the growth of bulbous species. For garlic plants regenerated *in vitro*, sucrose is an important factor in inducing bulb formation (Chaidir *et al*., 2023; Greedharry *et al*., 2024; Taha, 2017).

In vitro studies with other bulbous species of genus *Lilium* (Liliaceae) also report that during bulb formation the accumulation of starch is a fundamental step in the transition from shoot to bulb, and that synthesis of this compound is probably initiated by the change from apoplastic to symplastic sucrose discharge, which may be related to sucrose depletion (Wu *et al*., 2021).

Considering the hypothesis that sucrose is related not only to the energy supply of the culture medium but also to the process of bulb formation in general, the aim of this study was to examine the incidence of hyperhydricity and biometry following the transition from sprout to bulb in seedlings of the 'Ito' and 'Quiteria' garlic cultivars *in vitro*,in a culture medium with sucrose at concentrations of 0%, 2%, 4%, 6%, 8% and 10%.

MATERIAL AND METHODS

The experiment was conducted in the tissue culture laboratory in the district of Cristalina, Goiás, Brazil. Virus-free garlic microbulbs were obtained as per Torres *et al*. (2001) using garlic bulbs vernalised in a cold room at 4 ºC to a visual dormancy index of 80%. The bulbils then underwent thermotherapy by oven-drying at 37 ºC for an average of 35 days.

When the apices of the microbulbs reached 10 to 20 mm in size, they were disinfected in a 0.5% sodium hypochlorite solution for 20 minutes, then washed in distilled water and autoclaved. The meristems were excised under aseptic conditions in a laminar flow chamber and inoculated into the culture medium for regeneration and shoot induction (Initiation Phase). This was followed by the promotion of plant growth, rooting and the induction of bulb formation (Bulbing Phase).

Regeneration and shoot induction

The meristems were regenerated in a culture medium of MS salts (Murashige; Skoog, 1962), with the addition of 3% sucrose, 0.2% gerilte, 100mg L^{-1}i-inositol, 2.0 mg L^{-1} glycine, 1.0 mg L^{-1} thiamine HCl, 0.5 mg L^{-1} pyridoxine HCl, 0.5 mg L^{-1} nicotinic acid, 0.1 mg L^{-1} isopentenyladenine, 0.1 mg L^{-1} of indolebutyric acid and gelled with agar (0.6%). The pH of the culture medium was adjusted to 5.8 before autoclaving at 1.5 atm for 15 minutes.

A volume of 15 mL of the MS culture medium was added to sealed flasks ensuring there was no gas exchange. The explants were kept in a growth chamber for 30 days at a temperature of 25 ºC ± 2 ºC under cool-white LED lamps with a light intensity of 62 µmol m^{-2} $s^{-1}$and a photoperiod

Figure 1 - Colour rating scale for different degrees of hyperhydricity in garlic (*Allium sativum* L.) *in vitro*

Appearance					
Grade	1	2	3	4	5
Color	Dark green	Light green	Light brown	Brown	Purple
Hyperhidricity	Very high	High	Medium	Low	Zero

of 16 hours. Following shoot induction, the explants were transferred to the culture medium to promote the resumption of microbulb growth.

Bulb formation and sucrose concentration

For each of the treatments, six explants at the initial stage of development were transferred to 500 mL transparent plastic pots (COPOBRAS) containing 60 mL MS medium supplemented with vitamins, myo inositol (100 mg L^{-1}), sucrose (6%), and naphthaleneacetic acid (0.2 mg L^{-1}) and gelled with agar (0.6%). The pH of the culture media was adjusted to 5.8. The flasks were then sealed with lids ensuring there was no gas exchange.

During bulb formation, the flasks containing the seedlings were kept in a growth room for 180 days under the same conditions of temperature and photoperiod used during the regeneration phase.

A completely randomised design was used to evaluate the effect of the sucrose; the six sucrose concentrations (0%, 2%, 4%, 6%, 8% and 10%) were added to the culture medium of two cultivars ('Ito' and 'Quiteria'), resulting in a 6 x 2 factorial, with ten repetitions.

During this phase, each experimental plot comprised one 500 mL transparent plastic pot (COPOBRAS) that had a lid with no filters and contained six explants. After 180 days the following variables were analysed: number of bulbs per plant (NBP); percentage of plants showing bulb formation (%B); root fresh weight (RFW), shoot fresh weight (SFW) and bulb fresh weight (BFW), in grams, determined using a precision balance (Quimis); bulb diameter (BD) in millimetres, measured with a calliper; the Pearson correlation (dimensionless); and percentage of hyperhydricity, evaluated by colour using a rating scale proposed by the authors.

Hyperhydricity was determined by assigning a grade between 1 and 5 based on the rating scale. Grade 1 was assigned to plants with a green-coloured bulb, Grade 5 to plants with a purple-coloured bulb, and Grades 2 to 4 to plants coloured light green, or light or dark brown. Micropropagation requires plants that produce the greatest number of microbulbs, which should have a score of between 4 and 5, i.e. brown or purple in colour (Figure 1).

The resulting data were submitted to the F-test for analysis of variance at a significance of 0.05, and evaluated based on the assumptions of homogeneity of variances (Levene) and normality of residuals (Shapiro Wilk). When significant, the mean values of the variables were compared using Tukey's test and the Pearson correlation.

For treatments where the assumptions were not met even after transformation, the Kruskall-Wallis test, which does not require these assumptions, was applied. Each of the analyses were carried out using the R Software (v 3.5.1), adopting a significance level of 0.05.

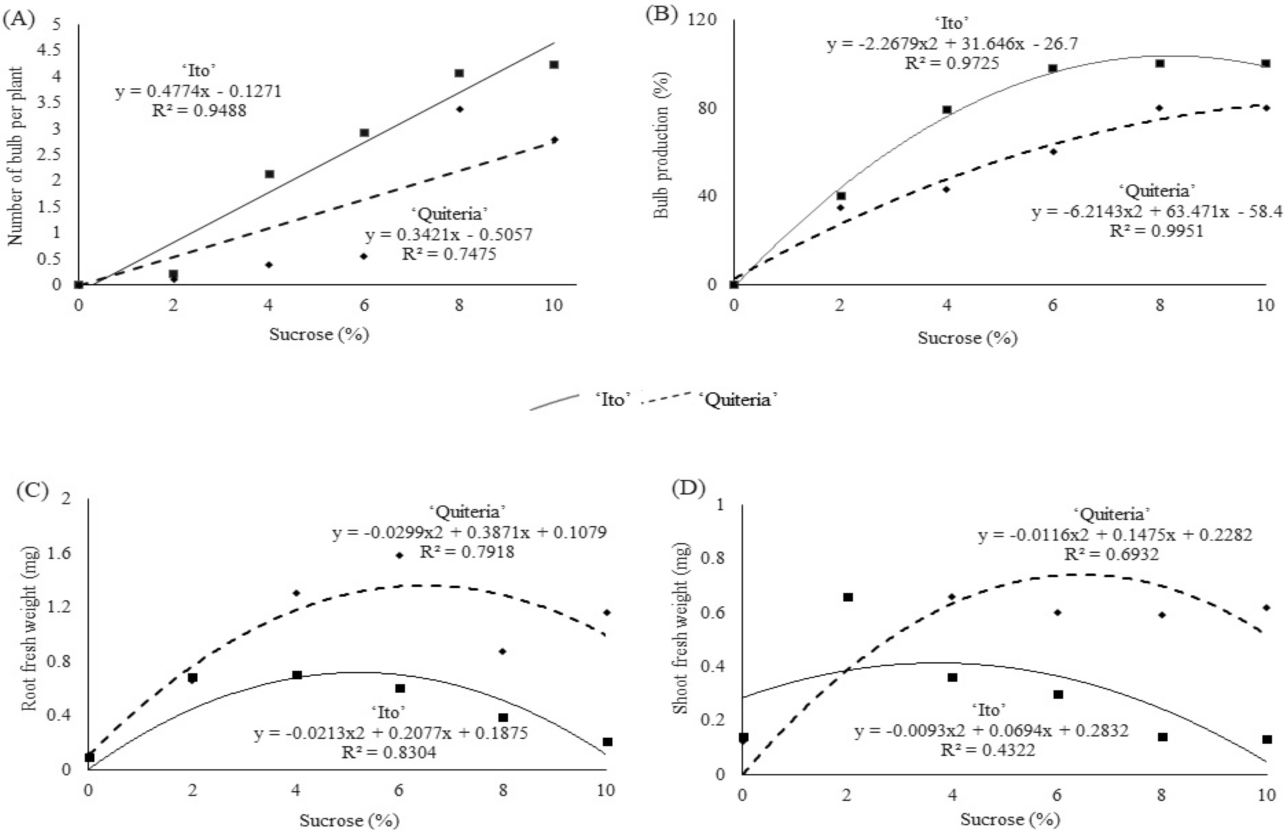
RESULTS AND DISCUSSION

As the plants present relatively limited photosynthesis, sucrose is an essential compound for *in vitro* plant development and is one of the most commonly used carbohydrates in the preparation of nutrient media, its most usual concentration varying from 2% to 3%. It is, however, occasionally used at higher concentrations, as in the case of embryo cultivation, bulblet induction in garlic, or root formation in cassava (Taha, 2017).

There was an interaction between the factors under test (Figure 2A). As the sucrose concentration in the culture medium increased to 10%, there was an increase in the number of microbulbs for both the 'Ito' cultivar, with a rate of 0.4774 bulbs for every 1% of added sucrose, and the 'Quiteria' cultivar, with a lower rate of 0.3426 bulbs. The production of a large number of bulbs per plant is important from an economic point of view, as the bulb is the end product.

Higher concentrations of sucrose (9%) in the culture medium of lily bulbs *in vitro* also proved to be an important factor for the formation, growth and regeneration of bulbs, while lower concentrations promoted the differentiation of shoots into roots (Zhang; Jía, 2014).

Figure 2 - Effect of sucrose concentrations (0%, 2%, 4%, 6%, 8%, 10%) on the variables A- number of bulbs per plant (NBP), B- percentage of plants showing bulb formation (%B), C- root fresh weight (RFW), and D- shoot fresh weight (SFW) in garlic (*Allium sativum* L.) seedlings of the 'Ito' and 'Quiteria' cultivars *in vitro*. 1) Significant at 5% by F-test. R^2: function adjustment



In terms of percentage bulb formation (Figure 2B), the 'Ito' cultivar showed a higher rate than 'Quiteria', even at lower sucrose concentrations. This is possibly related to the genetic characteristics of the cultivars since this behaviour has also been found in other studies (Pasupula*et al*., 2024; Resende *et al*., 2013).

In terms of root fresh weight, it was found that the 'Quiteria' cultivar was superior to 'Ito', producing roots with a weight of up to 1.36 mg in a culture medium with a concentration of 6.6% sucrose. In contrast, for the 'Ito' cultivar, the highest root fresh weight of 0.69 mg was achieved in a medium comprising 4.9% sucrose (Figure 2C). With regard to shoot fresh weight, the 'Quiteria' cultivar again had a higher mean weight, reaching 0.69 mg at a concentration of 6.5% sucrose, while for 'Ito', the meanweight was 0.41 mg in a culture medium with 3.7% sucrose (Figure 2D).

There was no difference in diameter between the two cultivars (Figure 3). The mean diameter was greater when *in vitro* cultivation was carried out in a medium containing 7.9% sucrose, resulting in a mean bulb diameter of 5.40 mm. Garlic seeds with larger diameters are desirable, as they have more reserves to undergo the next phase of seedling formation in a protected environment before being transplanted to the field.

These findings show that sucrose plays a very important nutritional role, also acting as a signalling molecule in bulb development. The sucrose molecule is composed of fructose and glucose;the use of increasing concentrations of the monosaccharide fructose and glucose, both individually or in an equimolar mixture, also stimulated greater production and greater bulb diameter in wild garlic (*Allium ochotense*) bulbs (Lee; Jeong, 2018).

Similarly, Hao *et al*. (2024) suggested that during *in vitro* bulb formationin *Lilium lancifolium*, sugars can induce cell division and cause bulb swelling, thereby increasing the bulb diameter during cultivation in a medium with a high concentration (9%) of sucrose. Bulb formation is thought to originate in the axillary meristematic cells of the leaves that divide and differentiate. The authors also note that sugars rapidly accumulate in the axillary buds, promoting their release following the loss of the stem tip, thereby having a more direct and rapid effect on the growth and development of the axillary organs.

There was also no difference between the cultivars for bulb fresh weight; it was estimated that 9.57% sucrose resulted in the highest mean bulb weight of 0.35 mg (Figure 3). This increase in bulb diameter and weight is desirable from a practical point of view, as the production of larger microbulbs generally avoids losses during acclimatisation, the next stage of *in vitro* plant micropropagation.

These results are in line with those described by Greedharry *et al*. (2024), who found that the number of bulbs per plant, percentage bulb formation, bulb diameter and fresh bulb weight increased as the concentration of sucrose in the culture medium increased to 9%. However, in the study by Longo *et al*. (2012) on micropropagation and *in vitro* bulb formation in garlic, the best response in terms of bulb formation and bulb fresh weight was at a sucrose concentration of 5%.

Although 'Quiteria' had fewer bulbs and lower percentage bulb formation, the plants of this cultivar presented higher values for root and shoot fresh weight than those of 'Ito', with a mean root fresh weight of 1.36 mg for 'Quiteria' and 0.69 mg for 'Ito' at a sucrose concentration of 6.4% and 4.9%, respectively; while the highest mean value for shoot fresh weight was 0.70 mg for 'Quiteria' and 0.41 mg for 'Ito' at a respective sucrose concentration of 6.4% and 6.7%.

Knowing the ideal amount of sucrose and its effect on biometrics is important, as excess sucrose in the culture medium can increase the production costs of *in vitro* cultivation. This is not because of the cost of the raw material, since this is relatively low, but because it increases the risk of *in vitro* contamination. Sucrose in the culture medium can favour the growth of such pathogens as fungi and bacteria, leading to competition between the plants and the microorganisms in the culture medium, and to the possible death of the plants (Gao *et al*., 2024).

According to Greedharry *et al*. (2024), elevated levels of carbohydrates affect the formation and growth of storage organs. To explain this, the author hypothesises that an increase in carbohydrates leads to an increased supply of energy, which can be used directly for the induction and growth of bulbs. He further comments on the alternative or complementary hypothesis that an increase in carbohydrate levels would lead to an increase in the osmolarity of the medium, resulting in stress that would favour the induction of storage organs.

On the other hand, Liu *et al*. (2017) suggests that the supply of carbohydrates may be more important than osmolarity for inducing bulb formation, and may even contribute to osmotic preparation for cryopreservation.

To determine whether there was a relationship between the characteristics of the plant itself (RFW and SFW) and the agronomic area of interest, which are the bulbs the plant produces (NBP, BD and BFW), the data were submitted to Pearson correlation analysis. The result is shown in Figure 4.

The correlation intensity was considered weak when *r* values were between 0.20 and 0.50, strong between the values of 0.50 and 0.70, and very strong between 0.70 and 1.0. Therefore, the closer to one (regardless of sign), the greater the degree of linear statistical dependence between the variables, while the closer to zero, the weaker the relationship.

Figure 3 - Effect of sucrose concentrations on the bulb fresh weight (BFW) and bulb diameter (BD) in garlic (*Allium sativum* L.) seedlings of the 'Ito' and 'Quiteria' cultivars *in vitro*. 1). Significant at 5% by F-test. R2: function adjustment

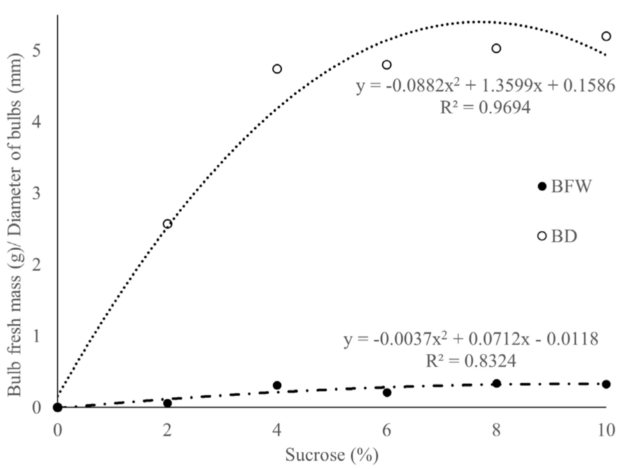
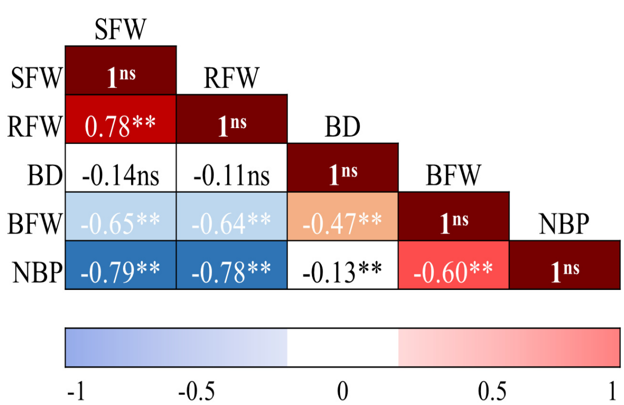


Figure 4 - Pearson correlation for shoot fresh weight (SFW), root fresh weight (RFW), bulb diameter (BD), bulb fresh weight (BFW), and number of bulbs per plant (NBP) in garlic (*Allium sativum* L.) seedlings *in vitro*



**Significant at 1% probability by t-test, ns Not significant at 1% probability by t-test

Analysis of the Pearson correlation showed that there was a strong negative correlation between the garlic plants for the vegetative characteristics RFW and SFW and the reproductive characteristics NBP and BFW. Based on the characteristics and correlations under analysis, the 'Ito' cultivar proved to be superior for large-scale *in vitro* cultivation due to its desirable agronomic characteristics. Therefore, for future research, it is suggested that experiments be carried out to reduce the proportion of shoot and root vegetative parts and increase the characteristics of interest in relation to the bulb. A study of hormonal balance or the addition of different osmotic agents to the culture medium could be one example of such a study.

Analysing the degree of hyperhydricity, the 'Ito' cultivar showed the highest quality (purple) for microbulbs produced at a sucrose concentration of 10%, while 'Quiteria' (Fig. 5) showed more positive results at concentrations of 8% to 10%, achieving a high percentage of microbulbs of Grades 4 and 5 (low hyperhydricity).

Figure 6A shows the green colour of the bulbs with hyperhydricity and figure 6C the smaller quantity produced in the culture medium containing only 2% sucrose.

According to the literature, adding sucrose for the *in vitro* cultivation of *Cochlospermum regium* (Bixaceae) resulted in a reduction in the percentage and intensity of hyperhydricity as the sucrose concentration in the culture medium increased to 9% (Palma *et al*., 2011).

Greedharry *et al*. (2014) found a reduction in the fresh weight of *Allium sativum* microbulbs produced at a higher sucrose concentration (12%). It was also reported that the mean values for number of bulbs per plant, percentage bulb formation, bulb diameter and bulb fresh weight were higher in media containing sucrose and fructose up to a level of 9%. However, the same behaviour was not seen for the number of bulbs per plant, which presented a maximum value of 1.6 bulbs in a medium containing 3% sucrose (Greedharry *et al*., 2024).

Figure 5 - Average percentage values of the scores assigned based on the degree of hyperhydricity in bulbs from garlic (*Allium sativum* L.) seedlings of the 'Ito' and 'Quiteria' cultivars *in vitro* submitted to concentrations of 2%, 4%, 6%, 8% and 10% sucrose added to the culture medium. Grade 1 was assigned to plants with a green-coloured bulb, Grade 5 to plants with a purple-coloured bulb, and Grades 2 to 4 to plants coloured light green, or light or dark brown. Mean values followed by the same letter do not differ statistically by Tukey's test at 5% probability

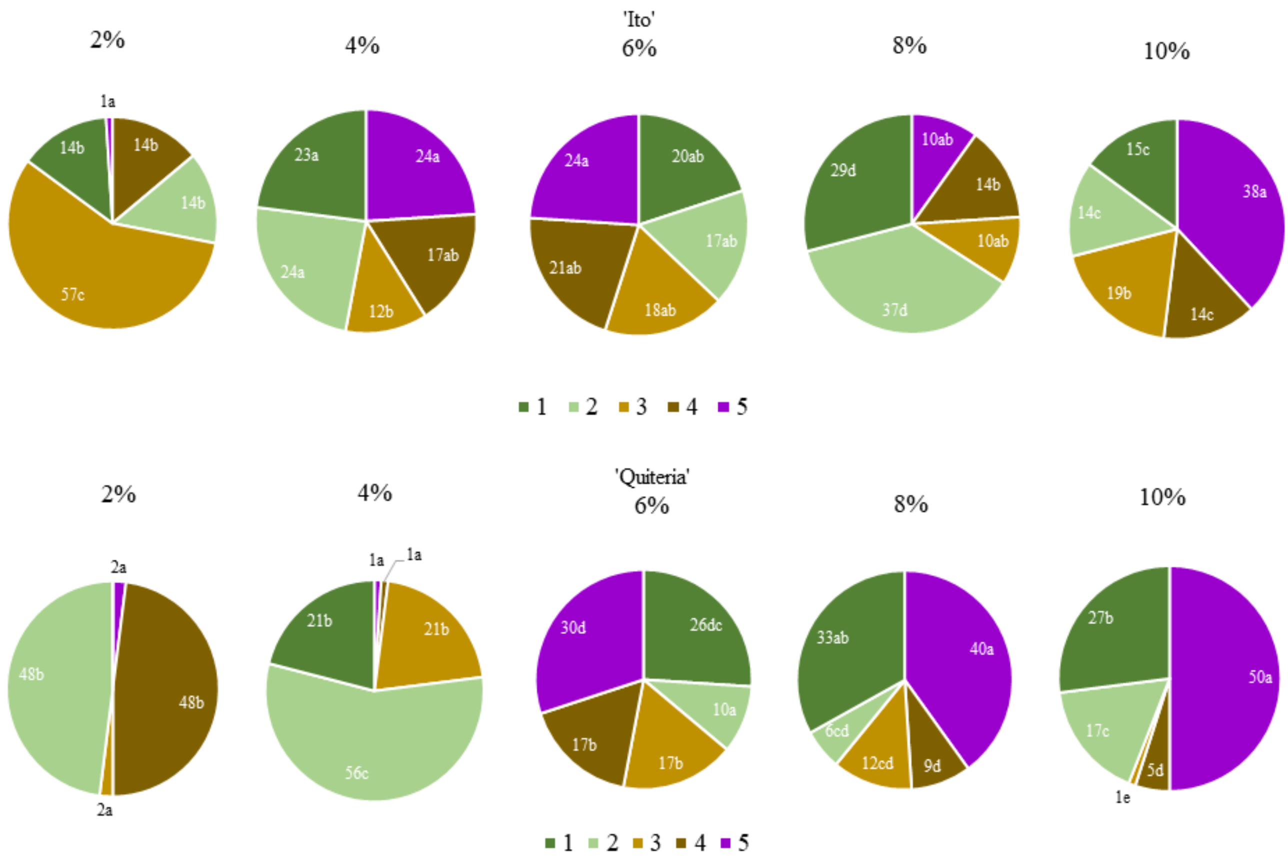
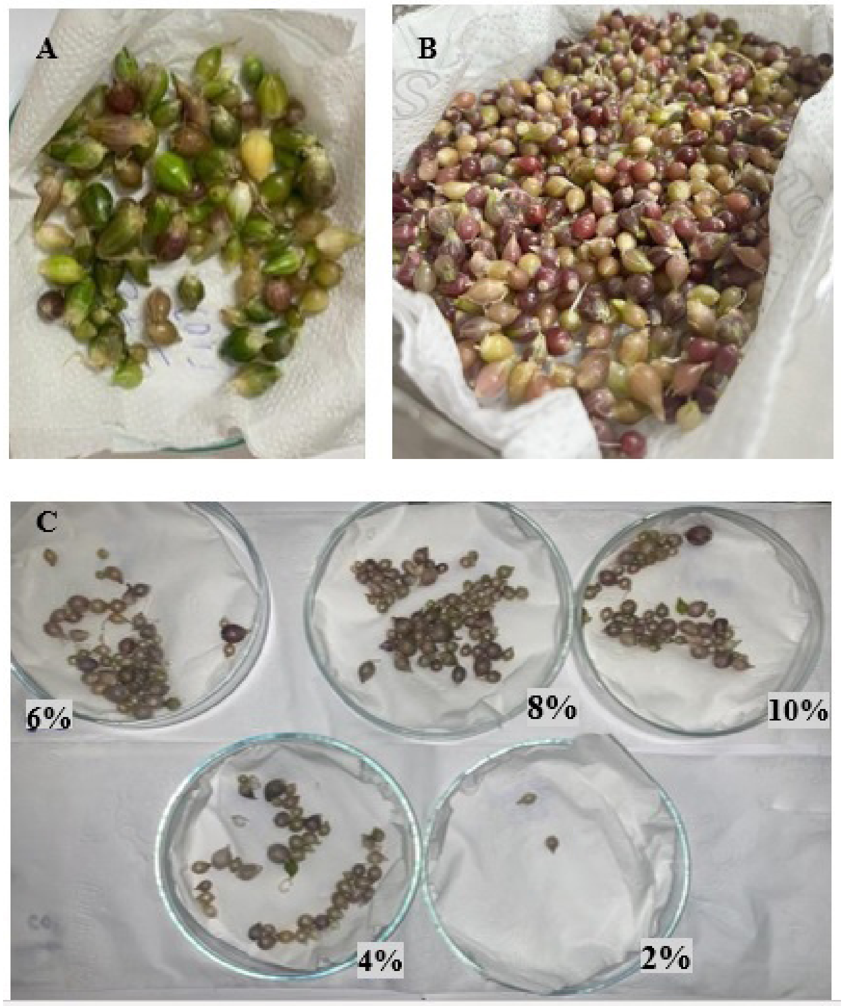


Figure 6 - Bulbs of seed garlic (*Allium sativum* L.) showing the green colour (A) of bulbs with hyperhydricity and the brown (B) colour of normal bulbs. Bulbs of the ‘Quiteria’ cultivar produced in culture media with 2%, 4%, 6%, 8% and 10% sucrose (C)



In some species, sucrose concentrations greater than 10% can reduce the osmotic potential (Ψs) of the culture medium, leading to a drastic reduction in the water potential (Ψw) of the explant and making it impossible to transfer the plant from the *in vitro* environment to an *ex vitro* environment (Aluko *et al*., 2021; Greedharry *et al*., 2024). In view of this, it is believed that an increase in the sucrose concentration to 8% or 10% can be used as an additional tool to reduce hyperhydricity and allow better development of the explants.

CONCLUSIONS

1. During bulb formation, a sucrose concentration of 10% for the ‘Ito’ cultivar and 8% and 10% for the ‘Quiteria’ cultivar afforded an increase in productive characteristics and resulted in a lower degree (purple) of hyperhydricity in the bulbs;
2. The vegetative characteristics of fresh root and shoot weight show a strong negative correlation with the productive characteristics of number of bulbs per plant and fresh bulb weight.

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