Agave sp. IAC4: a new and productive cultivar in plant tissue culture

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ABSTRACT

Considering climate change and Brazilian socio-regional aspects, Agave is a potential biomass-to-biofuel alternative with a great potential to be inserted in the Brazilian semi-arid region. However, its long-life cycle, monocarpy, and poor seed viability represent challenges for its establishment as a reference energy crop. Thus, plant tissue culture is a suitable technique for quickly obtaining plants able to meet commercial demand. *Agave* sp. IAC4 is an access from the Agave breeding program developed by the Agronomic Institute of Campinas, that until now, never been cultivated in vitro. Therefore, this study aims to present the first report of successful micropropagation of *Agave* sp. IAC4, using 3 different growth regulators. We observed that IAC 4 was able to successfully grow through direct organogenesis and embryogenesis in all media tested. The plantlets were able to be multiplicated and well acclimated in organic substrate with a survival rate of 80%.

Keywords: Micropropagation. Agave sp. Tissue culture. Plant development. Biofuel.

1 INTRODUCTION

Renewable energy development is a global, immediate need and the use of biomass to biofuels has proven to be an excellent alternative. Since the 1970s, Brazil has been making extensive use of biofuels, which has prevented the emission of 1.34 billion tons of CO2 due to the use of bioethanol[1]. Although sugarcane is the gold standard for obtaining ethanol, the gradual increase in global temperatures and changes in the rainfall cycle directly affect sugarcane plantations, reducing their productivity[2], leading to an immediate need of a more resilient source. Considering climate change and Brazilian socio-regional aspects, Agave is a potential alternative to be inserted in the Brazilian semi-arid region. Of Mexican origin, Agave is highly resistant to heat, capable of growing in a semi-arid climate, with good biomass production using relatively few resources[3]. It has a high accumulation of non-structural soluble carbohydrates[4], and its crassulacean acid (CAM) metabolism contributes to the low demand for water. However, its long-life cycle, monocarpy, and poor seed viability represent challenges for its establishment as a reference energy crop[5].

Agave propagation is mainly carried out using shoots and bulbils, but this practice is not ideal for large-scale production due to the possibility of disease transmission. Furthermore, obtaining seedlings on a large scale, at affordable prices, with quality assurance is an essential step in establishing Agave as a safe bioenergy resource. Thus, plant tissue culture is a suitable technique for quickly obtaining plants on a large scale that are free of pathogens and able to meet commercial demand. Although well known, the technique requires continuous improvement and optimization when aiming to cultivate new species. From a Brazilian origin the Agave sp. IAC4 is an access from the Agave breeding program developed by the e Agronomic Institute (IAC, Campinas) throughout hybridization[6]. Although it's interesting industrial characteristics such as size and fiber content, until now there is no report for in vitro micropropagation of its cultivar. Therefore, this study aims to present the first report of successful micropropagation of Agave sp. IAC4.

2 MATERIAL & METHODS

Aerial bulbils from mature IAC4 plants were collected, their leaves were removed and the interior was disinfected using detergent for 5 minutes, 70% alcohol for 3 minutes and 2.5% sodium hypochlorite for 20 minutes. Between each step, the bulbils were rinsed with sterile distilled water and after exposure to hypochlorite, 3 rinses were performed. Then, the bulbils were sliced transversely into at least 5 slices around 2 mm and introduced into Murashige and Skoog basal culture medium[7] (MS salts 4,44 g/L, sucrose 30g/L, agar 6,5 g/L, pH 5.8) containing 6-Benzylaminopurine (BAP) 44,3 µM + 2,4-Dichlorophenoxyacetic acid (2,4D) 0,11 µM, or BAP 44,3 µM + Indole-3-butyric acid (IBA) 0,49 µM, or BAP 44,3 µM alone. 20 bulbils were inserted in each of the 3 different culture media. The explants were observed periodically for 88 days and their growth was measured. Oxidation of the slices, origin of shoot (organogenesis or embryogenesis), presence or absence of callus were also observed. The seedlings were then transferred to MS medium without growth regulators, where they remained for 47 days until rooting. Then, the seedlings were acclimatized in organic substrate, in a greenhouse with shade and periodic irrigation. Graphs and statistics were performed using GraphpadPrism software. To compare means, Tukey Multiple Comparison test followed by two-way ANOVA were used, p value lower than 0,05 was considered significant.

3 RESULTS & DISCUSSION

After introduction, the seedlings had their growth monitored for 88 days, no significant difference was observed between the size of the seedlings maintained in the different culture media, with all culminating in a similar average size (figure 1 A). The number of shoots was also observed, but no significant difference was detected between the conditions. Regarding seedling development, growth was observed through direct organogenesis where the culture medium containing 2,4D auxin proved to be more efficient, with an average of 2,9 plants per bulbil (figure 1B) and 50 seedlings in total (table 1). The use of 2,4D also promoted a greater amount of callus, with an average of 3,2 calluses per bulbil. Regarding oxidation, the slices introduced in only BAP had a higher degree of oxidation, with 2,5 events per bulbil and 30 events in total (figure 1B). In these cases, the oxidized slices were not able to generate seedlings.

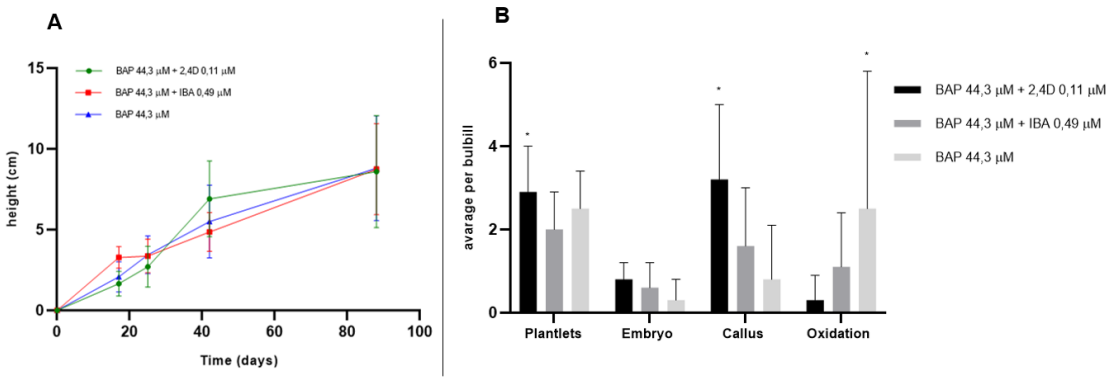

Figure 1 Seedling growth (A) and responsiveness (B) to different growth regulators. *p<0.05.

Literature reports the obtaining of seedlings of several Agave species, both through direct and indirect organogenesis (with an intermediate callus phase)[3,8]. The use of the cytokinin BAP and the auxins 2,4D and AIB have also been reported for other species in different quantities[8,9,10,11]. It is important to highlight that the balance of growth regulators is essential for the establishment and maintenance of seedlings in tissue culture. For the Agave genus, several combinations, varying between regulators and their quantities have already been reported, with different results, which highlights the fact that the technique is genotype-dependent[9,10,11].

Table 1 Quantity of direct organogenesis, direct embryogenesis and oxidation events in each culture medium tested

Growth regulator	Organogenesis event	Embryogenesis event	Oxidation
BAP 44,3 44,3 µM + 2,4D 0,11 µM	50	13	5
BAP 44,3 µM + IBA 0,49 µM	30	9	17
BAP 44,3 µM	23	4	30
total	103	26	52

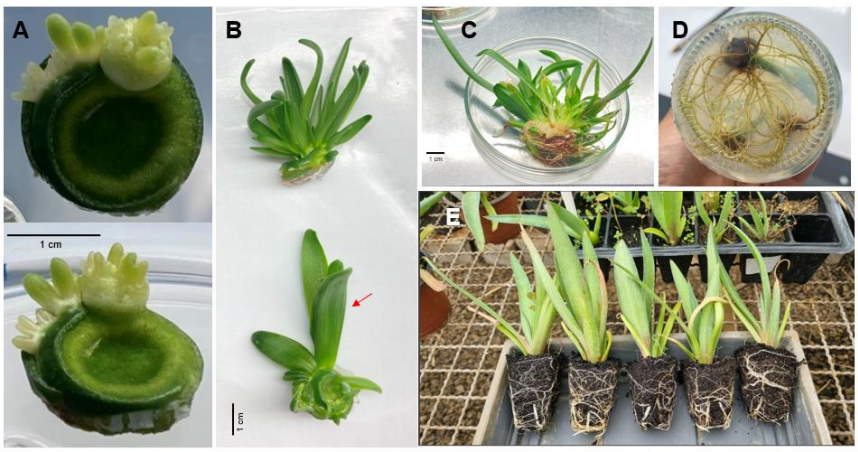

Figure 2 Overview of seedling development during the study. A: Embryos growing in BAP 44.3 44.3 µM + 2.4D 0.11 µM after 25 days. B: developing embryo (upper plant) and direct organigenesis (lower plant, red arrow), plantlets grown in BAP 44.3 44.3 µM + 2.4D 0.11 µM after 42 days. C: Longitudinal section of a developed embryo, after two subcultures, after 88 days. D: Well-developed roots in MS medium, after 47 days. E: plants acclimatized after 90 days in organic substrate.

In this work, the direct obtaining of somatic embryos using 2,4D stands out, where 13 events with somatic embryogenesis were obtained (table 1). The embryos have an initial globular and torpedo appearance (figure 2A), as previously reported in other agave species[12,13]. Embryos and seedlings by direct organogenesis (figure 2B, arrow) had sustained growth in the same culture medium. The embryos did not need to change culture conditions for their maturation and subsequent development (figure 2B, upper plan), diverging from previous studies for Agave[12,13]. Figure 2C shows the longitudinal section of a fully developed embryo, where it is possible to notice the polarity of development, with well-defined root and shoot, characteristics previously reported in the literature[14]. The embryos of Agave sp. IAC4 presented dozens of individual sprouts of different sizes, ranging from 2 to 13 cm, an average of 24 seedlings per developed embryo. Around 47 days in MS media without growth regulators days it was already possible to observe well developed and robust roots, enabling acclimatization (figure 2D). 133 seedlings were acclimatized and monitored. After 60 days 107 well-developed plants remained, resulting in a success rate of approximately 80%. Although the rate is slightly lower than other studies in the area, it is considered a good establishment rate for a new cultivar in tissue culture.

4 CONCLUSION

Agave sp. IAC4 is a cultivar that stands out for its size and possibility of supplying both the fiber and ethanol chains, in addition to being a cultivar developed in Brazilian territory. Its introduction in tissue culture proved to be feasible and with good prospects for multiplication and obtaining high quality seedlings. The use of 2,4D as auxin proved to be more interesting than IBA for obtaining greater quantities of seedlings. Aiming for scaling and optimization, other concentrations of 2,4D, as well as other auxins, should be tested. The use of bulbils made it possible to obtain somatic embryos directly, which is very interesting from an industrial point of view, as seedlings develop in large quantities and in a uniform manner from them. Rooting took place without the use of growth regulators, which is important for reducing costs and complexity of the process, facilitating future scaling. In view of the present results, it is concluded that *Agave* sp. IAC4 appears to be a plant with great potential for in vitro multiplication, able to meet the production chains of which it will be part in the future.

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