



Integrated TIS RITA[®] and PGRs strategies for enhanced in vitro propagation of carob (*Ceratonia siliqua* L.)

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Abstract

Ceratonia siliqua L., or carob, is an evergreen Mediterranean tree valued for its environmental and economic benefits, including soil fertility enhancement and low maintenance requirements. Conventional propagation methods, such as seeds and grafting, have limitations, highlighting the need for large-scale in vitro techniques, including Temporary Immersion Systems (TIS). In this study, we compared two culture systems, solid culture medium (SCM) and TIS RITA[®], for seedling development. TIS RITA[®] demonstrated superior growth, with higher shoot multiplication rates, longer shoot lengths, and greater fresh weight. It also showed better vigor without callus formation, whereas SCM exhibited some callus at the hypocotyl base. First, we investigated the specificity of zeatin's effect on carob by comparing it with blueberry shoots. We used the combination of zeatin and a CKX inhibitor to show the specificity of the effect. For carob, 0.2 mg/L zeatin with the CKX inhibitor resulted in the highest shoot induction and fresh weight, while blueberry shoots showed optimal regeneration with 1 mg/L zeatin and the CKX inhibitor. However, the addition of the CKX inhibitor in carob does not show bigger advantages with respect to its absence in terms of shoot regeneration, like the case of the increment of zeatin concentration. As a consequence, other plant growth regulators (PGRs) could be used to substitute this expensive PGR. Shoot multiplication experiments using different PGRs (BAP, *m*TR, *m*T and *o*TR) improved regeneration efficiency and revealed that TIS RITA[®] produced higher shoot numbers and weights compared to SCM. The longest shoots were obtained under specific PGRs concentrations, and morphological differences were observed between the two systems, with TIS RITA[®] generating more elongated shoots. Overall, these findings demonstrate the potential of in vitro techniques, particularly TIS RITA[®] and PGRs application, to improve carob propagation.

Key message

Temporary Immersion Systems is suitable for *Ceratonia siliqua* L. in vitro culture, and the use of cytokinin, not only zeatin, improves shoot quality and biomass production in this species.

Keywords *Ceratonia siliqua* L. · PGRs · CKX inhibitor · Zeatin · Temporary Immersion System (TIS)

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Introduction

Ceratonia siliqua L., commonly known as carob, is a woody sclerophyllous species of the Fabaceae family. This evergreen, long-lived, and slow-growing tree is native to the Middle East (Retana et al. 1994) and is widely distributed across the Mediterranean basin, where it forms part of the Oleo-Ceratonion plant communities that include several rare and paleoendemic species (Gianguzzi et al. 2022). Carob is generally dioecious; however, considerable sexual variability exists, with hermaphrodite and polygamous forms contributing to the wide diversity of cultivars (Romano et

al. 2002; Thomas et al. 2024). The species is recognized as a valuable multipurpose tree, providing both ecological and economic benefits (Catarino 1993).

Ecologically, carob is a pioneer species capable of improving soil fertility and facilitating the establishment of other plants in degraded and semi-arid areas. It thrives in marginal soils and under extreme climatic conditions, thereby contributing to reforestation in regions unsuitable for agriculture (Cavallaro et al. 2021). Its adaptability, growth plasticity, and longevity classify it as an agro-sylvo-pastoral species with potential contributions to climate change mitigation through long-term carbon sequestration (Martins-Loução et al. 2024).

Economically, carob pods and seeds have multiple uses, including as a chocolate substitute, in bioethanol production, and for locust bean gum (LBG, E-410) as a food additive. Other applications include sugar syrups, bioactive compound extraction (polyphenols and carotenoids), and livestock fodder (Bhat and Khan 2011; Loullis and Pinakoulaki 2018; Saci et al. 2020; Germec et al. 2021; Palaogianni et al. 2022; Yalimkaya and Özdemir 2022). Its canopy and foliage also make it suitable as an ornamental or shade tree, due to its tolerance to drought and air pollution and its low maintenance requirements (Shahzad et al. 2017; Thomas et al. 2024).

The growing demand for carob-based products, particularly the expanding carob bean gum market (Tzatzani and Ouzounidou 2023), underscores the need for efficient propagation systems to support large-scale cultivation. Considering this demand, along with the ecosystem services and wide range of economically valuable products derived from this crop, the development of large-scale *in vitro* propagation systems is essential to overcome the limitations of traditional propagation methods. Furthermore, the lack of structured nursery practices and limited genetic improvement programs are increasing the vulnerability of this species to genetic erosion, as observed in other neglected and underutilized crops (Martins-Loução et al. 2024).

Currently, carob cultivation relies primarily on seed propagation, cutting, and grafting (Romano et al. 2002; Gubbuk et al. 2020). Seeds exhibit physiological dormancy caused by a hard coat, resulting in low and irregular germination. Pretreatments such as chemical or mechanical scarification and soaking in warm water are often applied to improve germination. However, seed propagation does not ensure the maintenance of desirable genotypic traits, and dormancy further limits large-scale production programs (Barracosa et al. 2007; Cavallaro et al. 2021). In addition, seed-derived plants often delay fruit production because of the species' slow maturation (Martins-Loução et al. 2024). Conversely, seed dormancy represents an adaptive mechanism that promotes persistent soil seed banks, animal-mediated dispersal,

and survival in fire-prone Mediterranean ecosystems (Ortiz et al. 1995; Piotto et al. 2003).

Cutting propagation is hindered by low rooting rate, limiting large-scale cultivation (Gubbuk et al. 2011). Grafting enables clonal propagation of elite genotypes, but it is labor-intensive, costly, and highly dependent on nursery conditions (Batlle Caravaca and Tous Martí 1997; Martins-Loução et al. 2024). Overall, these techniques are not sufficient to meet the rising market demand and the need for expanded carob cultivation (Romano et al. 2002).

In this context, *in vitro* culture represents a promising strategy for clonal propagation, genetic conservation, and large-scale production of elite varieties and rootstock lines (Romano et al. 2002; Shahzad et al. 2017). Clonal micropropagation also preserves desirable bio-agronomic traits (Osório et al. 2012; Soumare et al. 2021). Among *in vitro* techniques, temporary immersion systems (TIS) offer advantages over semi-solid media, including a more natural environment, partial automation, reduced labor costs, and improved shoot and seedling development (Georgiev et al. 2014). TIS-propagated plants also exhibit higher adaptability during acclimatization, a critical phase for woody species (Preece 2010; Arencibia et al. 2013).

Cytokinins are key plant growth regulators (PGRs) that promote shoot multiplication *in vitro* (George et al. 2008; Avalbaev et al. 2012). In addition to PGR concentration, their stability can also be modulated to prolong activity while reducing undesirable side effects at high levels. The activity of an enzyme capable of irreversibly degrading cytokinins was first demonstrated in tobacco culture by Paces et al. (1971) and later by Whitty and Hall (1974), who named it cytokinin oxidase. The enzyme was subsequently reclassified as cytokinin dehydrogenase (CKX) after other electron acceptors (e.g., quinones) were identified as essential for its activity (Galuszka et al. 2000; Frebortova et al. 2004). CKXs catalyze the irreversible degradation of cytokinins through oxidative cleavage of the side chain at the sixth position of the purine ring, producing adenine/adenosine and the corresponding side-chain (Brownlee et al. 1975; Frebort et al. 2011; Wang et al. 2014). The side chain of cytokinin must have a double bond to guarantee the functioning of the enzyme in isopentenyladenine, zeatin, and their ribosylated derivatives (Zhang et al. 2011). CKXs have been characterized in several species, including *Hordeum vulgare* (Galuszka et al. 2000), *Triticum aestivum* (Frébort et al. 1999; Galuszka et al. 2001), *Beta vulgaris* (Kevers et al. 1997), and *Petunia hybrida* (Auer et al. 1999). Only a few gene families encoding CKXs have been identified, and their number varies across plant species (Zalabák et al. 2014). CKXs are FAD-dependent enzymes containing an *N*-terminal FAD-binding domain and a *C*-terminal cytokinin-binding domain (Dabravolski and Isayenkov 2021).

Because adenine acts as an inhibitor in various metabolic pathways, CKX concentration in plant tissue culture are generally low, complicating molecular characterization, in vivo studies, and protein isolation (Frebort et al. 2011; Ashihara et al. 2018).

If CKXs are the enzymes that catalyze the irreversible degradation of cytokinins, N-glucosyltransferases that promote N7 and N9 glycosylation of cytokinins are important in the irreversible inactivation of these PGRs (Frebort et al. 2011; Sakakibara 2025). CKXs and N-glucosyltransferases work together to modulate the activity of cytokinins.

Inhibition of CKXs through chemical or molecular approaches results in cytokinin accumulation in plant tissue, enhancing grain yield (Ashikari et al. 2005; Zalewski et al. 2010), stress tolerance, and delay in senescence (Nisler et al. 2021); moreover, it improves the regenerative capacity and in vitro organogenesis of explants in the micropropagation process (Chen and Wei 2018; Mazri et al. 2018; Nisler et al. 2021, 2022, 2024).

For example, CKX inhibitor number 52 reduced the degradation of exogenous iP in tobacco leaf disks and improved tobacco shoot regeneration (Nisler et al. 2022). CKX inhibitors no. 19 and 21 (Nisler et al. 2021) were shown to stimulate 2iP-induced direct somatic embryogenesis in *Coffea arabica* (Murvanidze et al. 2021).

In this work, we investigated the effect of a newly described diphenylurea-derived CKX inhibitor, 1-(3-chloro-5-trifluoromethoxy-phenyl)-3-[2-(2-hydroxy-ethyl)-phenyl]-urea (also known as compound 20), reported by Nisler et al. (2021), which shows promising potential for enhancing shoot regeneration in different species.

Several tissue culture protocols have already been developed for carob (Romano et al. 2002; Naghmouchi et al. 2012; Bouzdoudi et al. 2017; Georgiou et al. 2017; Shahzad et al. 2017; Saïdi et al. 2019; Nia et al. 2021; Ahmed et al. 2021), including TIS-based approaches (Cavallaro et al. 2017). Here, we present a simple and efficient method for aseptic in vitro germination of carob seeds without chemical scarification, followed by seedling establishment. From these seedlings, we compared the effects of the TIS RITA® and solid culture medium (SCM) on seedlings development (shoot tip number, length, and fresh weight) over sixty days, to maximize the source of explants (i.e., shoot tips). Shoot tips were then used to test the effects of compound 20, with or without zeatin, to evaluate its effectiveness in promoting carob shoot multiplication. *Vaccinium corymbosum* L. (blueberry) was included as a comparative control, given the species-dependent variation in regeneration efficiency reported for compound 20. To identify more effective alternative hormones or comparable to zeatin, we tested TIS RITA® and SCM culture in combination with four N⁶-substituted aromatic cytokinins: 6-benzylaminopurine (BAP),

meta-topolin (*mT*), *meta*-topolin riboside (*mTR*), and *ortho*-topolin riboside (*oTR*).

In conclusion, this study presents a comparative approach to carob micropropagation, aiming to identify optimal shoot regeneration protocols for large-scale in vitro cultivation of uniform rootstock clone lines, with potential application to elite varieties.

Results

Seed pre-germination, mechanical scarification, and germination rate

Pre-germination techniques and seed coat removal were applied to reduce polyphenol leaching into the MS medium, which can inhibit germination, impair seedling development, and promote seed dormancy (Kong et al. 2008; Inácio et al. 2013; Cavallaro et al. 2021). Polyphenol leaching was markedly reduced by replacing sterile distilled water on the 4th and 8th days of germination. This treatment resulted in rapid and uniform germination in Petri dishes, and seedlings developed normally in the MS medium in the absence of polyphenols.

No bacterial or pathogenic contamination was observed in seedlings by the 15th day of germination, indicating that pre-germination in Petri dishes did not lead to contamination issues.

Germination of mechanically scarified seeds (micropyle piercing technique) ranged from 0 to 10% within 50 h. Subsequently, germination increased sharply from 10% to 75% within 100 h. After 150 h, germination reached 90%, with the highest value (98%) observed after 200 h (8 days). At this point, nearly all seeds had developed a 2 mm radicle, and no further germination occurred (Fig. S1).

Comparison of carob seedling development in SCM and TIS RITA®

Fifteen days after germination, carob seedlings bearing ~1 cm shoot tips were transferred to two culture systems, SCM and TIS RITA®, to compare their development in the absence of PGRs. Significant differences were observed between the two systems in seedling performance, including shoot tip number, shoot tip length, and seedling fresh weight over a 60-day period (Fig. 1A).

After an additional 15 days of cultivation, RITA® vessels produced significantly more shoot tips per seedling (1.72 ± 0.66) than SCM (1.16 ± 0.38). Between 30 and 45 days, the number of shoot tips in TIS RITA® continued to increase from 1.88 ± 0.67 to 2.33 ± 0.90 , while SCM showed no significant change (1.22 ± 0.42 to 1.61 ± 0.50).

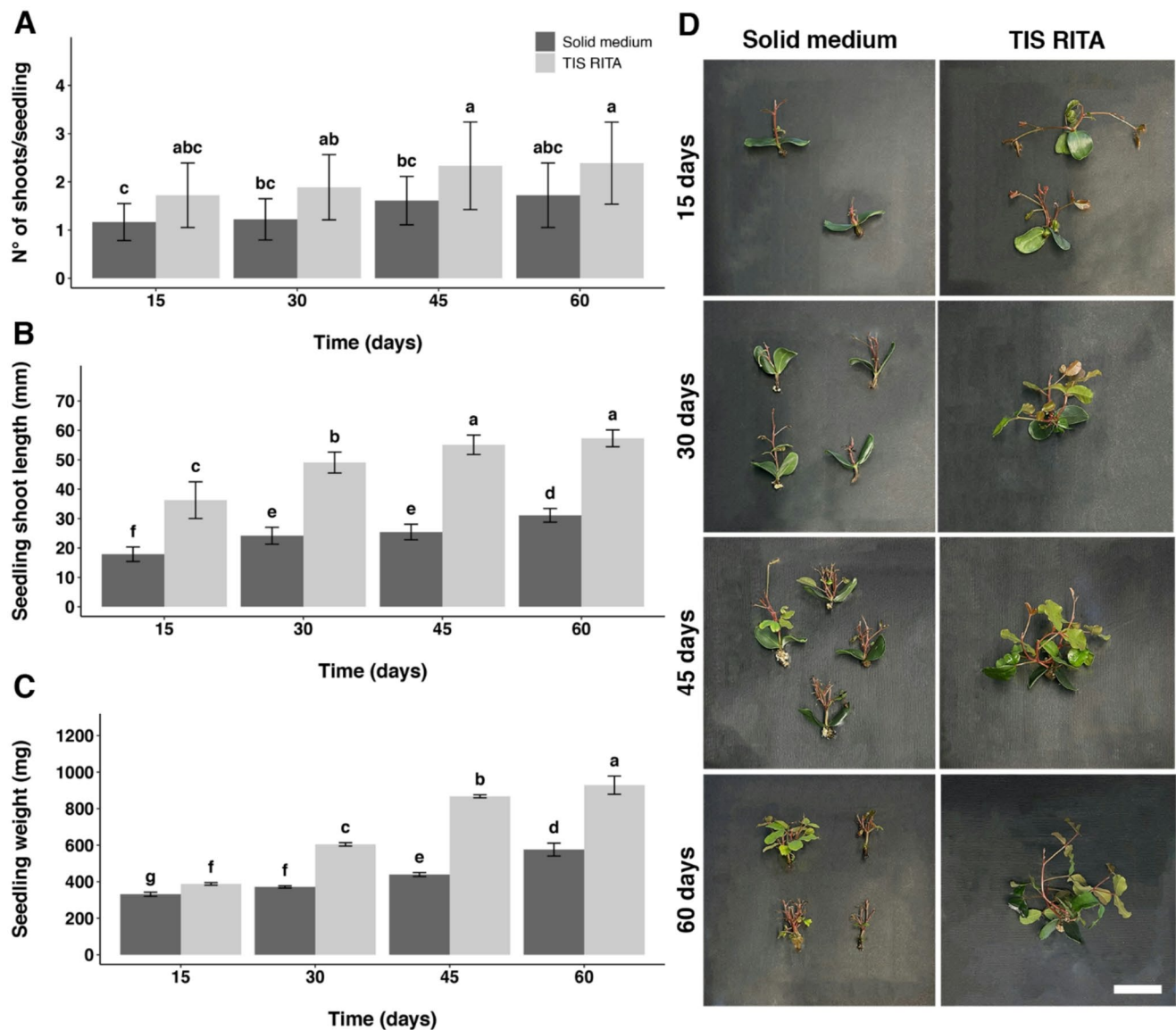


Fig. 1 Comparison of carob seedling development in SCM and TIS RITA[®]. **(A)** Number of shoots per seedling, **(B)** shoot length, and **(C)** shoot fresh weight after 15, 30, 45, and 60 days. **(D)** Morphological appearance of seedlings in SCM and TIS RITA[®] after 60 days. Scale

bar = 25 mm. Different letters within bars indicate significant differences. Data represent mean values $\pm$ standard deviation of three replicates, each consisting of 20 explants per culture system

After 60 days, the number of shoot tips in TIS RITA[®] stabilized at 2.38 ± 0.84 , showing no significant increase compared to values at 45 days. However, the final shoot multiplication rate remained higher than that of SCM (1.72 ± 0.66). Notably, SCM reached after 60 days the same number of shoot tips that TIS RITA[®] had already achieved by day 15 (Fig. 1A).

Significant differences in shoot tip length were evident after just 15 days, with TIS RITA[®] averaging 36.26 ± 6.24 mm compared with 17.86 ± 2.47 mm in SCM (Fig. 1B). In TIS RITA[®], the length of the shoot tips progressed from 49.06 ± 3.56 mm at 30 days to 55.10 ± 3.29 mm at 45 days. During the same timeframe, SCM showed minimal

length improvement, increasing from 24.16 ± 2.86 mm to 25.43 ± 2.63 mm. No additional improvements in the number of shoots were observed between 45 and 60 days. After 60 days, shoot tip in SCM measured 31.10 ± 2.32 mm, nearly half the length of those cultivated in RITA[®] vessels (Fig. 1B).

The increase in fresh weight was modest yet statistically significant after 15 days of cultivation, with averages of 331.20 ± 11.28 mg in TIS RITA[®] and 387.00 ± 7.06 mg in SCM. After 30 days, the variations in fresh weight among the seedlings became more pronounced, as RITA[®] vessels recorded an average of 604.30 ± 9.26 mg compared to 371.40 ± 5.85 mg in SCM. Over the next 45 to 60 days, TIS

RITA[®] demonstrated a consistent upward trend, increasing from 867.50 ± 7.95 mg to 928.60 ± 49.64 mg. Meanwhile, SCM also showed growth during the same period, with fresh weight rising from 439.30 ± 10.80 mg to 575.80 ± 35.33 mg (Fig. 1C).

Seedlings cultivated in TIS RITA[®] exhibited significantly greater vigor compared to those grown in SCM. Callus formation was absent in TIS RITA[®], while it was observed in SCM at the base of the hypocotyl between 30 and 60 days (Fig. 1D). The leaves of seedlings raised in RITA[®] vessels were not only larger but also more abundant; however, the leaves in SCM maintained a normal structure. Overall, neither culture system showed any signs of browning or hyperhydricity (Fig. S2).

Comparative study of the effect of CKX inhibitor 20 on the number of shoots, shoot length, fresh weight, and number of internodes in carob and blueberry cultured in SCM

Initial explants (shoots) were separated from 60-day-old seedlings (derived from the first experiment) and then incubated in both culture systems for shoot multiplication. Initial explants were selected carefully to ensure uniformity in size and weight of shoots (13 mm and 50 mg for carob and 20 mm and 40 mg for blueberry). In carob a notable synergistic effect was observed between zeatin at the lowest concentration (0.2 mg/L) and the CKX inhibitor at 0.37 mg/L, which resulted in enhanced shoot induction at 6.15 ± 0.83 (Fig. 2A). When the same concentration of CKX inhibitor was added to the higher zeatin concentration (1 mg/L), there was also an improvement in shoot multiplication (6.00 ± 0.57) compared to 1 mg/L of zeatin alone (5.52 ± 0.96), although it did not exceed the response achieved with the lower zeatin concentration. Zeatin alone, whether at 0.2 mg/L or 1 mg/L, successfully induced adventitious shoots; however, 0.2 mg/L elicited a better response (5.89 ± 0.56) than 1 mg/L (5.52 ± 0.96). Conversely, when the CKX inhibitor was applied alone at 0.37 mg/L, it failed to produce shoots (1.84 ± 0.37), a result similar to that of the control (1.10 ± 0.31) (Fig. 2A).

In blueberry shoots cultivated for 45 days under SCM, the effects of zeatin and the CKX inhibitor demonstrated varied responses (Fig. 2B). The combination of 1 mg/L zeatin and 0.37 mg/L CKX inhibitor produced the most effective results in terms of shoot regeneration, yielding an average of 12.66 ± 2.20 new shoots per explant. Conversely, when zeatin was applied at a lower concentration (0.2 mg/L) alongside 0.37 mg/L CKX inhibitor, shoot multiplication significantly decreased to 3.20 ± 0.40 , a result statistically comparable to the effect of zeatin at 0.2 mg/L alone (3.40 ± 0.62). The highest concentration of zeatin alone

(1 mg/L) demonstrated a moderate capability to stimulate new shoot formation, resulting in 8.66 ± 1.62 shoots. In contrast, the CKX inhibitor tested independently failed to promote new shoot formation, yielding only 1.13 ± 0.34 shoots, similar to the control conditions, which recorded 1.03 ± 0.18 shoots (Fig. 2B).

Carob shoot length was positively influenced by the addition of 0.37 mg/L CKX inhibitor and 0.2 mg/L zeatin, resulting in the highest recorded lengths (42.20 ± 10.68 mm), as well as with 1 mg/L zeatin (35.82 ± 14.45 mm) (Fig. 2C). Also in the absence of the CKX inhibitor, both concentrations of zeatin (0.2 and 1 mg/L) showed an increase in shoot length (34.44 ± 8.18 mm and 30.27 ± 10.35 mm, respectively), with no significant statistical differences between them but less pronounced than in the case where the CKX inhibitor was present. Compared to the CTRL group (11.31 ± 1.07 mm), there was a slight improvement in shoot length with only the presence of CKX inhibitor (21.75 ± 2.23 mm) (Fig. 2C).

In blueberries, the effect on shoot length varied based on the presence or absence of CKX in relation to different concentrations of zeatin (Fig. 2D). Notably, the presence of the CKX inhibitor significantly enhanced the effectiveness of 1 mg/L zeatin, resulting in the highest measurement of 53.42 ± 4.83 mm. In contrast, this measurement decreased substantially without the CKX inhibitor, dropping to 41.46 ± 7.93 mm. Lowering the zeatin concentration to 0.2 mg/L led to a reduction in shoot length. However, when the CKX inhibitor was introduced alongside 0.2 mg/L zeatin, it had a positive effect on shoot length, increasing it to 44.40 ± 11.49 mm compared to 39.98 ± 8.57 mm with zeatin alone at 0.2 mg/L. The lowest shoot length measurements were recorded under the control condition at 39.66 ± 4.98 mm, as well as in the medium supplemented solely with the CKX inhibitor, which yielded a value of 39.84 ± 4.83 mm (Fig. 2D).

The fresh weight of carob shoots achieved the highest values when treated with CKX inhibitor combined with 0.2 mg/L zeatin (312.94 ± 17.47 mg), 1 mg/L zeatin (307.99 ± 12.85 mg), or 0.2 mg/L zeatin alone (307.24 ± 14.66 mg). No significant differences were noted among these treatments (Fig. 2E). An observed decrease in shoot fresh weight occurred with the increased concentration of zeatin to 1 mg/L (284.04 ± 8.32 mg). The control condition yielded a lower fresh weight (49.32 ± 1.24 mg), while the supplementation of CKX inhibitor alone resulted in a statistically significant, albeit modest, increase (81.40 ± 5.35 mg) (Fig. 2E).

For blueberry shoots, the highest fresh weight was recorded with the combination of 1 mg/L zeatin and 0.37 mg/L CKX inhibitor (1206 ± 120.71 mg) (Fig. 2F). Lower weights were observed for those treated with 0.2 mg/L or 1 mg/L Zeatin alone (407.60 ± 142.96 mg and

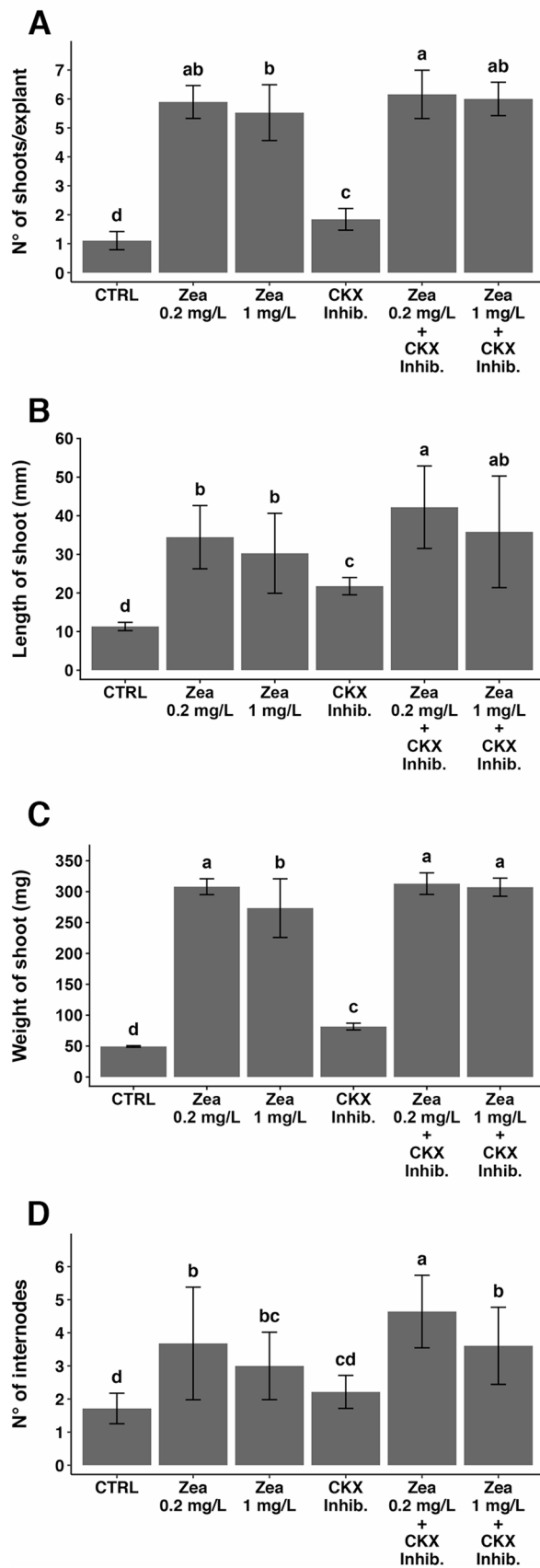
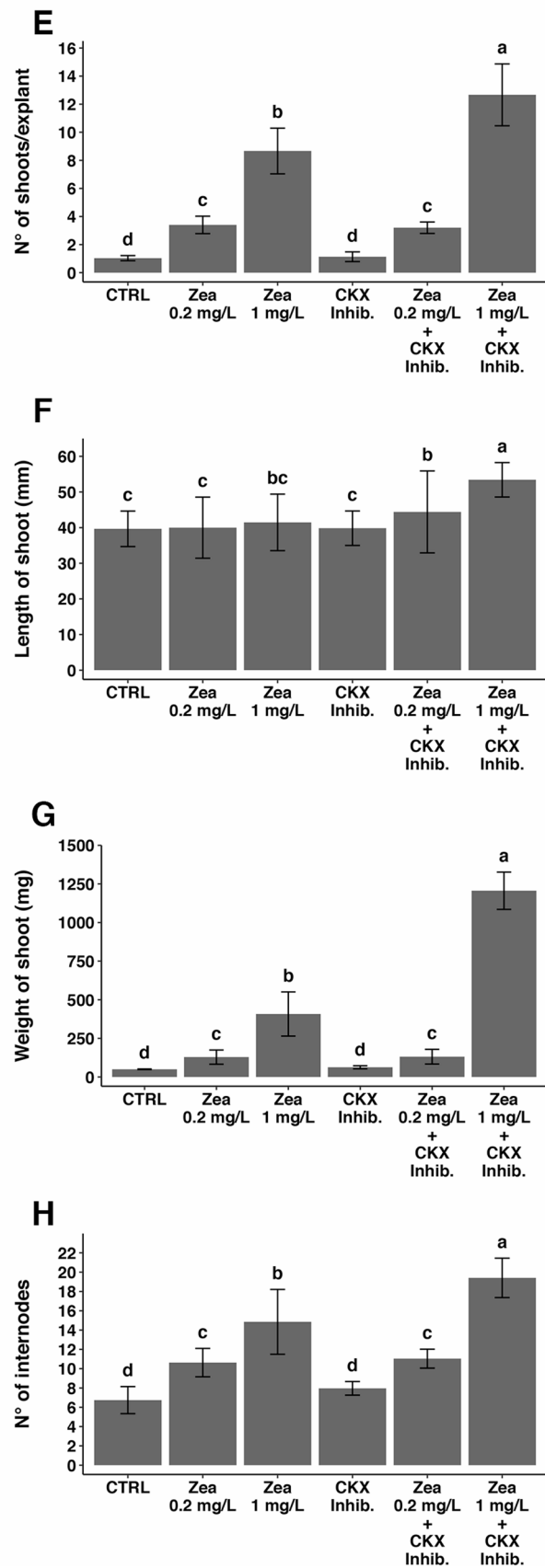
Ceratonia siliqua*Vaccinium corymbosum*

Fig. 2 Effect of zeatin alone (0.2 and 1 mg/L), CKX inhibitor alone (0.37 mg/L) or in combination (Zea 0.2+CKX inhibit 0.37 mg/L and Zea 1+CKX 0.37 mg/L) on shoot multiplication (A, E), length (B, F) fresh weight (C, G) and number of internodes (D, H) in carob (A–D) and in blueberry (E–H). Different letters within bars indicated significant differences. The data represent mean values $\pm$ standard deviation of three replications made up of 30 explants

128.40 $\pm$ 46.01 mg, respectively), as well as for the combination of 0.2 mg/L Zeatin and 0.37 mg/L CKX inhibitor (131.03 $\pm$ 47.49 mg). Overall, the CKX inhibitor did not enhance shoot fresh weight, yielding results comparable to the control (62.66 $\pm$ 9.82 mg and 49.63 $\pm$ 2.04 mg, respectively) (Fig. 2F).

The number of internodes varied significantly depending on the different media used for incubating carob shoots (Fig. 2G). The results indicated that the combination of 0.37 mg/L CKX inhibitor and 0.2 mg/L zeatin positively influenced internode production in the shoots, resulting in an average of 4.64 $\pm$ 1.09. A lower yet acceptable number of internodes was observed with CKX inhibitor and 1 mg/L zeatin, yielding an average of 3.60 $\pm$ 1.16; however, this was not significantly different from the results obtained with 0.2 mg/L zeatin (3.67 $\pm$ 1.70). Increasing the zeatin concentration to 1 mg/L did not improve the internode count but instead led to a decrease (3 $\pm$ 1.01). Furthermore, the CKX inhibitor alone did not enhance internode production, yielding results comparable to those of the control group (2.21 $\pm$ 0.49 and 1.71 $\pm$ 0.46, respectively) (Fig. 2G).

For blueberry shoots, the combination of CKX inhibitor and 1 mg/L zeatin resulted in an impressive internode production of 19.40 $\pm$ 2.04, followed by 1 mg/L zeatin alone, which produced an average of 14.85 $\pm$ 3.35 (Fig. 2H). Conversely, adding the CKX inhibitor to 0.2 mg/L zeatin did not yield an improvement in the number of internodes compared to zeatin alone at 0.2 mg/L (11.03 $\pm$ 0.97 versus 10.62 $\pm$ 1.47). Similarly, in this species, the CKX inhibitor alone did not demonstrate any stimulating effect on the number of internodes, with results closely matching those of the control group (7.96 $\pm$ 0.70 and 6.74 $\pm$ 1.40, respectively) (Fig. 2H).

At the conclusion of the 45-day incubation period, the morphology and development of shoots in both species were significantly influenced by all tested culture conditions (Fig. 3). In the case of carob shoots, cultivation in CTRL conditions or with the CKX inhibitor at 0.37 mg/L resulted in no new shoot production, maintaining only a single shoot that exhibited no active growth (Fig. 3A–B). Additionally, shoot tip necrosis was noted under both conditions (Fig. 3A–B).

In contrast, treatments with 0.2 mg/L zeatin and 0.2 mg/L zeatin combined with the CKX inhibitor resulted in increased vigor, longer shoots, active proliferation, and thicker stems in terms of diameter (Fig. 3C–D). However, the presence of

the CKX inhibitor occasionally led to foliar necrosis in the shoots (Fig. 3D). Notably, when zeatin concentration was raised to 1 mg/L, shoot growth was inhibited (Fig. 3E–F). Although new shoots were induced, they exhibited limited development, and some displayed tip necrosis. Interestingly, the addition of the CKX inhibitor resulted in healthier and longer shoots with normal morphology, where shoot tip necrosis did not occur (Fig. 3F). No signs of hyperhydricity were observed in shoots across all treatments (Fig. 3A–F).

Regarding blueberry shoots, those cultured without zeatin, irrespective of the CKX inhibitor's presence, exhibited smaller stems, reddish leaves, and lacked adventitious shoot proliferation (Fig. 3G–H). No instances of shoot tip necrosis were observed. The introduction of either 0.2 mg/L zeatin or 0.2 mg/L zeatin in conjunction with the CKX inhibitor led to a slight improvement in shoot regeneration and vigor (Fig. 3I–J). A consistent induction in plant proliferation was observed upon increasing the zeatin concentration to 1 mg/L (Fig. 3K–L). However, a marked increase in productivity and biomass production was evident when the CKX inhibitor was added to 1 mg/L zeatin (Fig. 3L), resulting in the proliferation of multiple shoots and new buds, characterized by taller and thicker stems along with healthy leaf morphology, all without any signs of hyperhydricity.

In vitro carob shoot multiplication in SCM and TIS RITA®

Initial explants (shoots) were separated from 60-day-old seedlings (derived from the first experiment) and then incubated in both culture systems for shoot multiplication. Initial explants were selected carefully to ensure uniformity in size (13 mm) and weight (50 mg) of shoots. In control (CTRL) conditions, the number of shoots did not differ significantly between SCM (1.08 $\pm$ 0.14) and TIS RITA® (1.16 $\pm$ 0.29); however, significant differences were observed when PGRs were supplemented (Fig. 4A). Overall, shoot multiplication in RITA® vessels was greater than in SCM, regardless of the PGR applied.

The highest shoot production in TIS RITA® was achieved with 0.5 mg/L BAP, averaging 5.00 $\pm$ 0.66 shoots, followed by 0.5 mg/L *mT* with 4.00 $\pm$ 0.66 shoots. A similar trend was observed in SCM, where the maximum number of shoots (3.41 $\pm$ 0.14) was also seen with 0.5 mg/L BAP, closely followed by 0.5 mg/L *mT*, which produced an average of 3.08 $\pm$ 0.14 shoots. The use of 0.5 mg/L *mTR* yielded comparable results in both culture systems, showing statistically different but similar averages: 2.33 $\pm$ 0.14 in SCM and 2.75 $\pm$ 0.25 in TIS RITA®. The lowest multiplication of shoots occurred with the addition of 0.5 mg/L *oTR*, resulting in averages of 1.33 $\pm$ 0.29 in SCM and 1.67 $\pm$ 0.29 in TIS

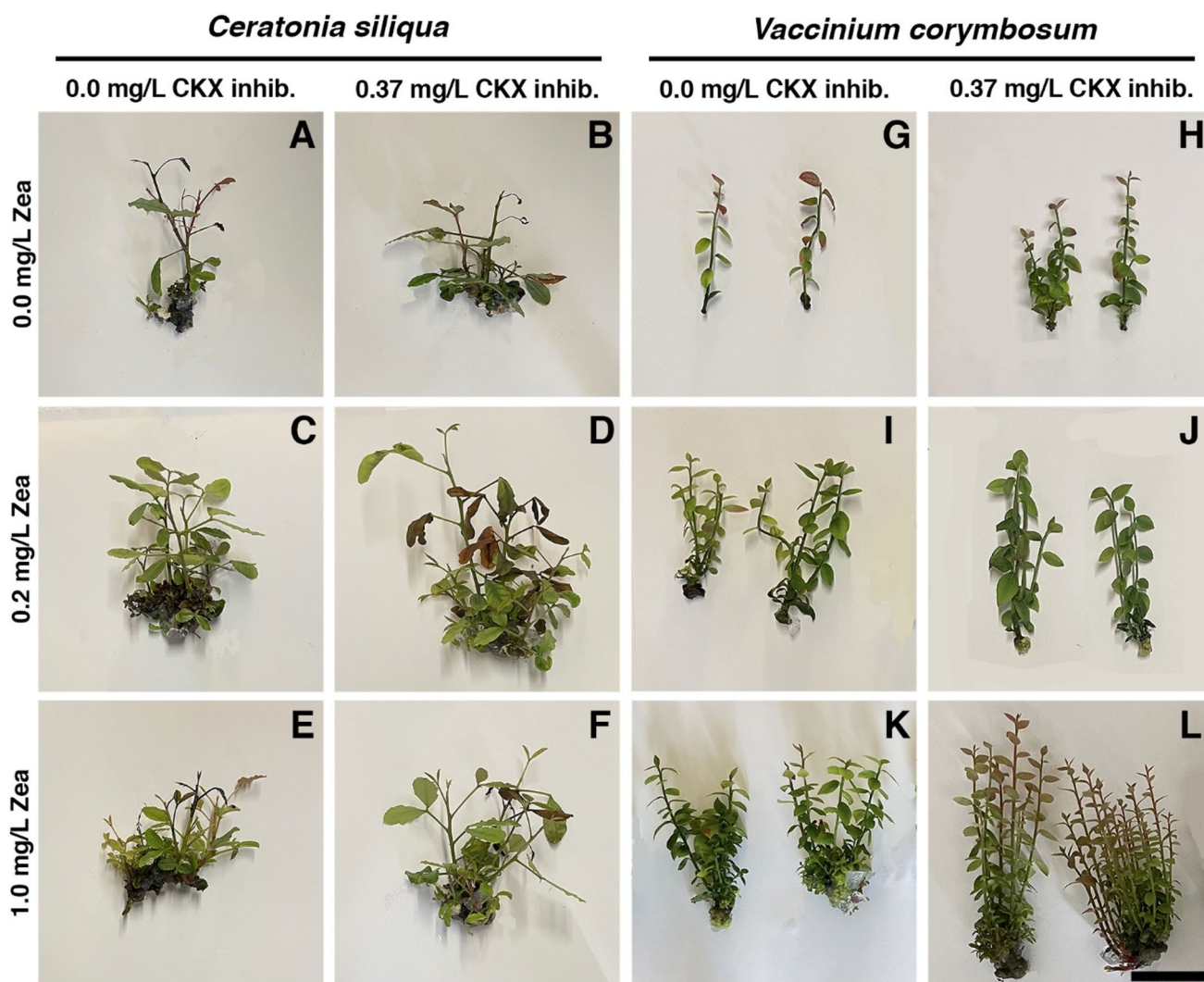


Fig. 3 Imaging of carob (A–F) and blueberry (G–L) shoots morphology and development after treatment without PGRs or with zeatin alone (0.2 and 1 mg/L), CKX inhibitor alone (0.37 mg/L) or in combi-

nation (Zea 0.2+CKX inhibit 0.37 mg/L and Zea 1+CKX 0.37 mg/L). Scale bar = 10 mm

RITA[®], which showed no significant differences when compared to the control (CTRL) group (Fig. 4A).

Significant differences were noted in shoot length across various culture systems with different plant growth regulators (PGRs) (Fig. 4B). Generally, the TIS RITA[®] system yielded longer shoots compared to the SCM system. Even under control conditions, TIS RITA[®] showed an average shoot length of 46.68 ± 2.45 mm, whereas SCM presented only 10.73 ± 0.73 mm.

The longest shoots were recorded in the TIS RITA[®] system with 0.5 mg/L *mTR*, measuring 79.47 ± 3.8 mm, followed closely by 0.5 mg/L BAP and 0.5 *mT*, which measured 76.31 ± 3.9 mm and 74.57 ± 2.26 mm, respectively. There were no significant differences between these two PGRs. The lowest shoot lengths were observed with

0.5 mg/L *oTR* (62.89 ± 2.82 mm), as well as under control conditions (46.68 ± 2.45 mm) (Fig. 4B).

In the SCM, the longest shoots were recorded with 0.5 mg/L *mT*, measuring 32.57 ± 1.86 mm, followed by 0.5 mg/L BAP at 26.57 ± 3.61 mm, 0.5 mg/L *mTR* at 21.57 ± 1.83 mm, and 0.5 mg/L *oTR* at 14.26 ± 0.73 mm. These values were relatively close to the CTRL condition, which measured 10.73 ± 0.73 mm (Fig. 4B).

Significant differences were also noted between the various culture systems combined with different plant growth regulators (PGRs) regarding shoot fresh weight (Fig. 4C). The highest shoot fresh weight was observed in TIS RITA[®] with 0.5 mg/L BAP, totaling 500.35 ± 3.65 mg, followed by 0.5 mg/L *mT* at 425.35 ± 5.96 mg and 0.5 mg/L *mTR* at 295.28 ± 3.31 mg, all showing statistically significant differences. In SCM, the highest fresh weight values

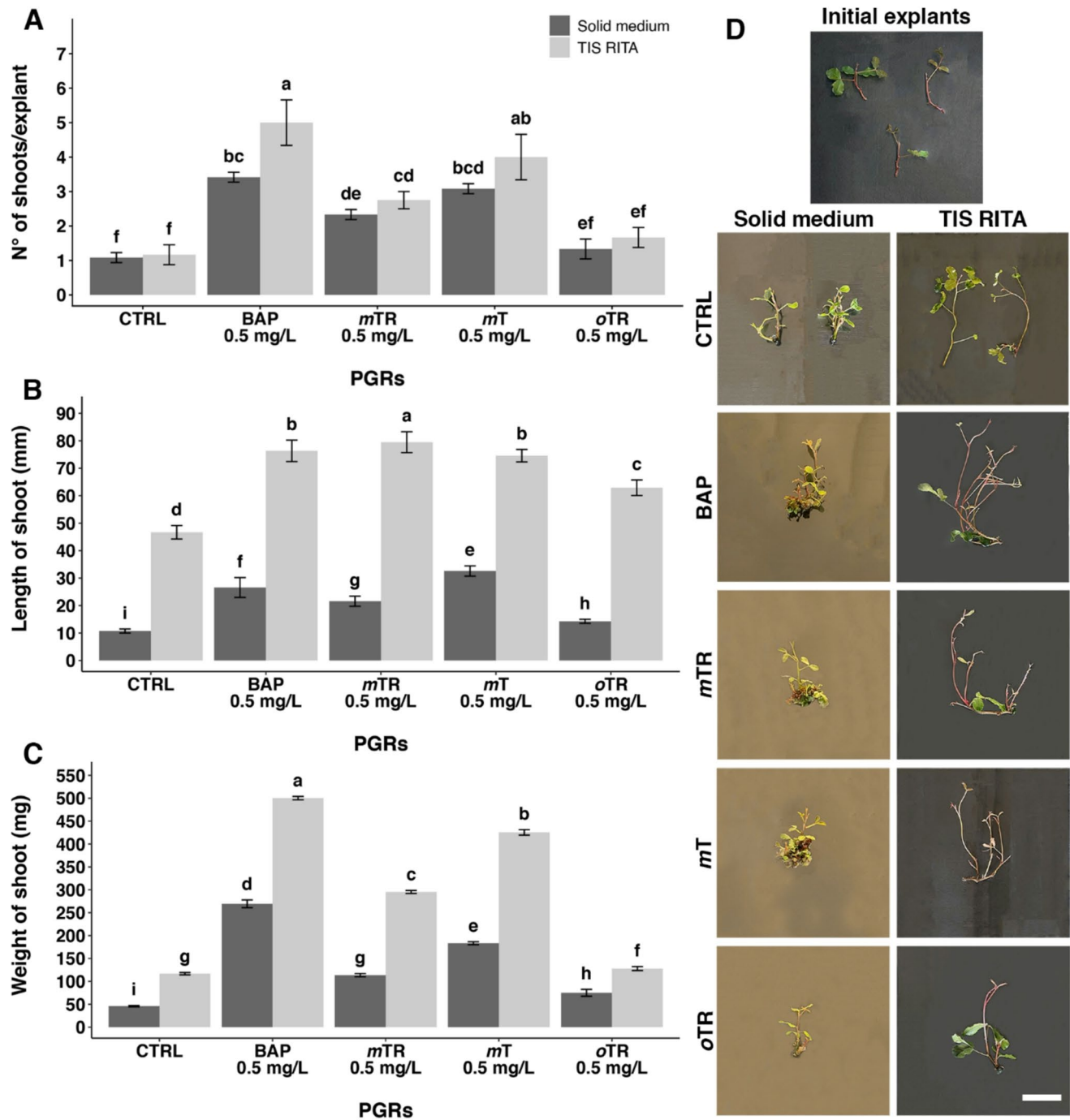


Fig. 4 In vitro carob shoot multiplication on SCM and TIS RITA[®] with BAP and three different topolins (*m*TR, *m*T, and *o*TR). (A) Number of shoots, (B) shoot length, and (C) shoot fresh weight produced in SCM and TIS RITA[®] with the same concentration of different PGRs. (D) Morphological appearance of shoots developed in SCM and TIS

RITA[®] within 45 days. Scale bar = 10 mm for SCM and 25 mm for TIS RITA[®]. Different letters above bars indicated significant differences. Data represent mean values $\pm$ standard deviation of three replications, each consisting of 30 explants per culture system

were recorded with 0.5 mg/L BAP (269.35 ± 8.41 mg) and 0.5 mg/L *m*T (183.42 ± 3.27 mg). Conversely, *o*TR did not effectively stimulate shoot fresh weight in either SCM (75 ± 7.59 mg) or TIS RITA[®] (127.92 ± 4.32 mg). Regarding the CTRL condition in RITA[®] vessels, the fresh

weight was comparable to that of 0.5 mg/L *m*TR in SCM (113.71 ± 3.38 mg) at 116.78 ± 2.57 mg, with no significant differences noted. In SCM, the CTRL condition exhibited the lowest fresh weight value at 45.85 ± 1.51 mg (Fig. 4C).

Over a period of 45 days, the appearance of the shoots changed significantly depending on the culture system utilized (Fig. 4D), demonstrating distinct morphological differences between SCM and TIS RITA[®], even under CTRL conditions (Fig. 4D). In RITA[®] vessels, shoots exhibited greater elongation, thinner stems, and a reddish coloration in comparison to those in SCM. Additionally, the horizontal orientation of the shoots in TIS RITA[®] reduced apical dominance, facilitating the emergence of lateral shoots. Conversely, shoots in SCM were more compact, less elongated, thicker, and displayed a greenish hue in contrast to those in TIS RITA[®]. Morphologically, shoots in the *o*TR condition appeared less developed across both culture systems when compared to other PGRs. Notably, callus formation was not observed in any of the PGR treatments or culture systems tested (Fig. 4D).

Active shoot growth was evident in RITA[®] vessels, accompanied by a notable level of adaptability, except under CTRL conditions and with *o*TR. Moreover, biomass density gradually decreased from the BAP condition to the *o*TR condition (Fig. S3).

Discussion

Carob propagation is attracting increasing attention, but it presents several limiting factors, starting with seed germination. It is influenced by physical dormancy that develops as an adaptive mechanism for increasing survival chances against abiotic stress and for allowing seed germination over time and space (Gunes et al. 2013; Klupezyńska and Pawłowski 2021). Seed dormancy is caused by the hard seed coat and can be alleviated through various treatments, including mechanical scarification, soaking in water at room temperature or in boiling water, immersing in sulfuric acid, soaking in gibberellic acid, or applying dry heat. (Ortiz et al. 1995; Pérez-García 2009; Cavallaro et al. 2021).

In the present study, a germination rate of 98% was achieved within 200 h through the mechanical scarification of seeds via micropile piercing. This finding aligns with the results reported by Pérez-García (2009), who observed a 99% germination percentage and identified mechanical scarification as the most effective method for enhancing germination in these species. Similarly, Gunes et al. (2013) highlighted mechanical scarification, combined with sulfuric acid treatment, as the most effective method for improving germination rates. Cavallaro et al. (2021) further demonstrated that seed dormancy is associated with the presence of polyphenols in the seed coat, which can have an inhibitory effect on germination.

To mitigate the negative impacts of the seed coat and polyphenols on germination, this study included the

removal of the seed coat and pre-germination washing prior to *in vitro* establishment. These techniques proved effective in minimizing the need to transfer seeds and seedlings to fresh medium every five days, as suggested by Shahzad et al. (2017).

Seedlings were utilized as a homogeneous source of explants to investigate their development and micropropagation in a temporary immersion bioreactor, in comparison to solid medium. The derived shoot tips were evaluated for the number of shoots, shoot length, and shoot fresh weight development efficiency through a comparative approach.

Carob culture establishment was achieved successfully in bioreactors (RITA[®]) despite their vulnerability to contamination due to the high nutrient availability and high oxygen exchange they provide (Lotfi and Werbrouck 2020). In addition, shoot tips-derived seedlings demonstrated a greater and easier adaptability to *in vitro* culture conditions, being immune to heavy contaminations characterizing explants from the field (Romano et al. 2002). Indeed, *in vitro* initiation from adult tree-derived axillary shoots or meristem tips can be time-consuming due to the high contamination rate (Brugaletta et al. 2009), seasonal influence on meristem harvesting (Naghmouchi et al. 2012), and phenolic compound self-toxicity (Romano and Louçao 1992).

TIS bioreactors, such as RITA[®], have been successfully applied for micropropagation of different species (Businge et al. 2017; Anglana et al. 2023; Capaci et al. 2024). This cutting-edge technique can avoid problems related to hyperhydricity, reduce labor costs, minimize energy requirements, and prevent carbon dioxide and ethylene accumulation in the culture vessel, offering a more natural growth environment (Georgiev et al. 2014). Nonetheless, the potential of bioreactors needs to be explored and optimized for each species and for each kind of explant within the species (Akdemir et al. 2014). This is the first study that investigates carob seedling development by using and comparing the SCM and TIS RITA[®] systems.

The RITA[®] bioreactors demonstrated significant advantages in terms of the number of shoots developed per explant, as well as in shoot length and fresh weight. Over a 60-day cultivation period, improvements were noted in all growth parameters when compared to the traditional solid medium. This advantage can be leveraged to enhance biomass production for further multiplication, thereby facilitating large-scale cultivation of carob rootstocks from the earliest stages.

To further investigate a novel strategy for improving shoot proliferation of carob, we tested the performance of zeatin alone and in combination with a new compound called 1-(3-chloro-5-trifluoromethoxy-phenyl)-3-[2-(2-hydroxy-ethyl)-phenyl]-urea (also known as compound 20). It belongs to a diversified group of inhibitors of cytokinin oxidase/dehydrogenase (CKX) that reduce the degradation

of cytokinins and could be exploited in plant tissue culture of relevant crops (Nisler et al. 2021, 2022). Two zeatin concentrations (0.2 and 1 mg/L) were tested to elucidate their effect on the number of shoots, shoot length, shoot fresh weight, and number of internodes in both carob and blueberry. Among the growth regulators, zeatin and 2-iP are the most used for micropropagation of blueberry (Arencibia et al. 2013; Correia et al. 2024), and in particular, zeatin has been demonstrated to enhance shoot multiplication and elongation in different *Vaccinium* species (Reed and Abdelnour-Esquivel 1991; Gajdošová et al. 2006; Debnath 2008; Fan et al. 2017; Schuchovski and Biasi 2019; Georgieva and Kondakova 2021; Sun et al. 2023).

In our study, we found that zeatin significantly improved growth parameters in both species, exhibiting dose-dependent responses. In carob, the lowest concentration of zeatin (0.2 mg/L) outperformed the highest concentration. Notably, the application of a CKX inhibitor alongside the 0.2 mg/L zeatin did not enhance the growth of in vitro plantlets, yielding results comparable to those obtained with 0.2 mg/L zeatin alone. However, the CKX inhibitor alone did not stimulate de novo shoot formation when applied without zeatin, confirming its zero intrinsic cytokinin activity. The data do not support a straightforward synergistic effect between zeatin and the CKX inhibitor in blocking zeatin degradation. In fact, the reduced effectiveness of 1 mg/L zeatin compared to 0.2 mg/L suggests that enhanced shoot development observed when the CKX inhibitor was added to 1 mg/L zeatin warrants further investigation.

Regarding blueberry, the highest concentration of zeatin proved to be superior for shoot proliferation. The addition of a CKX inhibitor to 0.2 mg/L zeatin did not significantly enhance growth; however, when applied to the higher concentration of zeatin, it further increased shoot proliferation and other growth parameters.

When applied alone, the CKX inhibitor did not stimulate blueberry shoot formation, confirming its lack of cytokinin activity while highlighting its crucial role in protecting zeatin from degradation. These results align with those of Nisler et al. (2022), who examined the same CKX inhibitor (compound 20) on tobacco leaf discs for shoot regeneration and found that the compound alone did not promote shoot induction, but it effectively protected 2-iP. A similar mechanism of action for the CKX inhibitor (compound 20) was observed in Canadian poplar leaf explants. As with the previous case, shoot regeneration was not achieved when the compound was applied alone; however, it did occur and was enhanced in the presence of 2-iP, showcasing its role as a cytokinin protector. (Nisler et al. 2022).

Zeatin appears to be a good candidate for improving shoot proliferation of carob, but the inefficiency of the CKX inhibitor suggests that zeatin could be replaced by

other molecules with lower cost, opening the possibility to test other PGRs that could have the same or better performance with respect to zeatin, like BAP, *mT*, *mTR*, and *oTR*. According to many authors (Brugaletta et al. 2009; Osório et al. 2012; El Bouzdoudi et al. 2017; Cavallaro et al. 2017; Saïdi et al. 2019) 0.5 mg/L BAP was found to be the most adequate cytokinin concentration for shoot multiplication of carob. Using this reference, we further investigated the effect of other PGRs such as *mTR*, *mT*, and *oTR* together with BAP in SCM and TIS RITA[®] culture system.

The study of the effect of PGRs on carob micropropagation confirmed that BAP, as reported in other studies (Romano et al. 2002; Brugaletta et al. 2009; Saïdi et al. 2019), has the tendency to be the most efficient for shoot multiplication and improvement of shoot fresh weight, when compared to *mT*, *mTR* and *oTR*.

On the other hand, we demonstrated that shoot elongation was also effectively promoted by *mTR* in TIS RITA[®] and by *mT* in SCM.

This study marks the first use of *mTR* for the micropropagation of carob. Our findings confirm that BAP perform better than hydroxybenzylaminopurines (topolins) in carob plant tissue culture. Previously, Shazad et al. (2017) demonstrated that shoot regeneration from carob cotyledonary nodes was higher with *mT* compared to Kn and TDZ; however, BA ultimately produced the best results. Similarly, Gentile et al. (2014) found that *mT* was less efficient than BA in enhancing shoot proliferation in *Prunus domestica* and *Prunus insititia*.

In other species, *mT* improved the frequency of shoot induction, for example in *Sesamum indicum* (Elayaraja et al. 2019), in *Eriocephalus africanus* (Madzikane-Mlungwana et al. 2017), in *Zantedeschia albomaculata* (Chang et al. 2003), in *Aloe polyphylla* (Bairu et al. 2007) and in *Aloe arborescens* (Amoo et al. 2012).

oTR bioassays reported in the literature show that its activity is generally lower than that of BAP and its *meta* derivatives (Holub et al. 1998; Doležal et al. 2006, 2007). Nonetheless, *oTR* has been identified in several plant species (Werbrouck 2010; Aremu et al. 2014; Grira et al. 2023). Aside from cytokinin bioassays, *oTR* has never been tested in micropropagation. For the first time, we report the effects of *oTR* in the in vitro culture of carob shoot tips. Our findings indicated that *oTR* produced detrimental effects on shoot proliferation, shoot length, and shoot fresh weight, making it unsuitable for the micropropagation of carob.

Regardless of the plant growth regulators (PGRs) used, the efficacy of TIS RITA[®] was superior in terms of the number of shoots, shoot length, and fresh weight when compared to SCM. The enhanced efficiency of the bioreactor can be attributed to the improved uptake of nutrients and PGRs from the entire shoot surface, including leaves via

stomata and aqueous pores, in contrast to the solid medium, where adsorption occurs primarily through the cut ends of the shoots (Guan and De Klerk 2000; Schonherr 2006). Other advantages of TIS include their capacity for automation and precise control over immersion frequency and duration, which reduces mechanical stress on the explants and preserves the morphological integrity of in vitro plantlets (Georgiev et al. 2014).

To date, there has been only one study on mass proliferation of carob from seedling apices by TIS, in which the number of shoots and shoot fresh weight were enhanced in the Plantform bioreactor compared to solid medium (Cavallaro et al. 2017). In addition, our study explored shoot multiplication using shoot tips isolated from 60-day-old seedlings and confirmed the potential of combining BAP and RITA[®] for large-scale cultivation of elite varieties in future scenarios.

This study examines several innovative parameters for carob in vitro culture, offering new perspectives for its future large-scale micropropagation.

Materials and methods

Seed collection, mechanical scarification, sterilization, pre-germination and in vitro initiation from seedlings

Mature pods from a 60-year-old wild carob genotype were collected in Santa Maria di Leuca, where this species grows naturally (39°48'21.3"N 18°21'21.2"E). Following the harvesting process, 120 seeds were extracted from the pods and stored at room temperature in a Petri dish. Prior to sterilization, the seeds underwent mechanical scarification as a dormancy-breaking treatment. Specifically, a small incision was made at the chalazal side of the seeds to pierce the micropyle, removing approximately 2 mm of the hard seed coat to facilitate imbibition.

This micropyle piercing was performed carefully to avoid damaging the radicle. The seeds were then sterilized using the following procedure: they were thoroughly washed under running tap water for half an hour, transferred to a 10% bleach solution for 20 min, and subsequently placed in a 3% (v/v) Plant Preservative Mixture[™] (PPM[™], Plant Cell Technology, USA) solution, where they were stirred for 24 h to ensure both additional sterilization and seed imbibition. After the sterilization process, pre-germination was carried out with the following procedure: seeds were placed in 9-cm Petri dishes (ten replicates of 12 seeds each i.e. 120 seeds) containing a single sterile filter paper moistened with 10 ml of sterile distilled water and sealed with Parafilm[®] (Fig. S4A). The germination temperature was 23±2 °C,

since it is considered the optimum for carob seed germination (De Michele et al. 1988). To avoid phenolic leaching, the seeds were transferred twice (at an interval of 4 days, on the 4th and 8th germination days) onto fresh, sterile distilled water, under the same above-mentioned conditions.

Germination was assessed when the radicle protrusion reached approximately 2 mm in length (Fig. S4B), and the number of seeds germinated was recorded daily (Cavallaro et al. 2021). The percentage of germination was calculated by dividing the total number of seeds germinated after eight days by the total number of seeds collected and multiplying by 100.

At the end of germination test, seedlings without seed coat (Fig. S4C) were inoculated in “De Wit” culture tubes (Duchefa Biochemie, Haarlem NL) containing 10 mL of hormone free culture medium supplemented with full-strength Murashige and Skoog (1962) mineral salts and vitamins, 30 g/L sucrose, 0.1% PPM[™], 0.735 g/L CaCl₂·2H₂O gelled with 0.7% (w/v) agar (Duchefa Biochemie, Haarlem NL) for fifteen days, in order to detect contaminations. The pH of the culture media was adjusted to 5.8 using 1 M KOH before autoclave sterilization. Seedlings were incubated at 23±2 °C under a 16-h photoperiod (60–90 µmol m⁻² s⁻¹ flux, cool white fluorescence light). After fifteen days of cultivation, carob seedlings with at least 1 cm of apex and a weight of approximately 160 mg (Fig. S4D) were transferred to two different culture systems (TIS RITA[®] and SCM) to evaluate seedlings' development in the absence of PGRs.

Carob seedlings development in SCM and TIS RITA[®] and data analysis

Vegetative apex from carob seedlings, each with a minimum apex length of 10 mm and an approximate weight of 160 mg, were transferred into two different culture systems (SCM[®] and TIS RITA[®]) to evaluate their development in the absence of plant growth regulators (PGRs). We compared two in vitro culture systems: in the SCM system, each seedling was cultured in a glass test tube (32×200 mm, round U bottom, Sigma Aldrich) containing 25 mL of full-strength MS medium, enriched with 30 g/L sucrose, 0.1% PPM, and 0.735 g/L CaCl₂·2H₂O, solidified with 0.7% (w/v) agar (Duchefa Biochemie, Haarlem NL). In the TIS RITA[®] system, seedlings were cultured on agar-free full-strength MS medium, containing the same mineral salts and vitamins, along with 30 g/L sucrose, 0.1% PPM, and 0.735 g/L CaCl₂·2H₂O. The explants were cultivated according to the manufacturer's instructions for the bioreactor (<https://www.vitropic.fr>). Each bioreactor housed six seedlings, which were immersed in a liquid medium (200 mL) for 2 min every 6 h using an aerating pump. Seedling

development was monitored with twenty explants per culture system and repeated three times. In both cultivation systems, the pH was maintained at 5.8, the temperature was kept constant at 23 ± 2 °C, and lighting was provided with a 16-hour photoperiod ($60\text{--}90 \mu\text{mol m}^{-2} \text{s}^{-1}$ flux, cool white fluorescent light). All media were autoclaved at 121 °C for 16 min. The parameters recorded after 15, 30, 45, and 60 days for both the SCM and TIS RITA® systems included the number of shoot tips per seedling, their length (mm), and fresh weight (mg).

Effect of CKX inhibitor compound 20 on shoot multiplication, shoot length, fresh weight and number of internodes of carob and blueberry in SCM

Effect of compound 20 on shoot multiplication of both species was tested in SCM; carob shoots were cultured in FULL GAS ECO2 Box (height 80 mm, base 125×65 mm, top and cover 150×90 mm, white filter for gas exchange) containing 100 mL of full-strength MS medium mineral salts and vitamins with 30 g/L sucrose, 0.1% PPM, 0.735 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ gelled with 0.7% (w/v) agar (Duchefa Biochemie, Haarlem NL). Blueberry shoots were cultured in FULL GAS ECO2 Box containing 100 mL of full-strength McCown's Woody Plant Medium (WPM) mineral salts and vitamins with 30 g/L sucrose, 0.1% PPM gelled with 0.7% (w/v) agar (Duchefa Biochemie, Haarlem NL). Control condition (CTRL) was compared with the supplement of selected concentrations of zeatin and compound 20 alone or combined: 0.2 mg/L zeatin, 1 mg/L zeatin, 0.37 mg/L compound 20 alone, 0.2 mg/L zeatin+0.37 mg/L compound 20 and 1 mg/L zeatin+0.37 mg/L compound 20.

Five shoots per box were incubated for a total 6 box/treatment. Shoot cultivation with the different treatment in SCM was repeated thrice.

Initial fresh weight and length of carob were 50 mg and 1.3 cm, respectively, whereas initial fresh weight and length of blueberry were 40 mg and 2 cm, respectively. In carob, the pH of culture was 5.8, while in blueberry, the pH of culture was 5 according to Fan et al. (2017).

The number of shoots was determined by taking the number of shoots at the end of each experiment and dividing by the initial explants cultured in SCM and TIS RITA®. Shoot length was also calculated together with the fresh weight. In addition, the number of internodes was calculated in both species.

The number of shoots per explant, shoots length, shoots fresh weight and number of internodes/shoot were recorded after 45 days of culture.

In vitro carob shoot multiplication in SCM and TIS RITA® and data analysis

For shoot multiplication, two different in vitro culture system were compared: in SCM shoots were cultured in FULL GAS ECO2 Box (height 80 mm, base 125×65 mm, top and cover 150×90 mm, white filter for gas exchange) containing 100 mL of full-strength MS medium mineral salts and vitamins with 30 g/L sucrose, 0.1% PPM, 0.735 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ gelled with 0.7% (w/v) agar (Duchefa Biochemie, Haarlem NL). In addition to the control condition (CTRL), the supplement of selected concentrations of PGRs was tested: 0.5 mg/L BAP, 0.5 mg/L *mT* (*meta*-topolin), 0.5 mg/L *mTR* (*meta*-topolin riboside), and 0.5 mg/L *oTR* (*ortho*-topolin riboside). Five shoots (approximately 2 cm) per box were incubated for a total of 6 boxes/PGRs.

In TIS RITA®, shoots were cultured on agar-free full-strength MS medium with 30 g/L sucrose, 0.1% PPM, and 0.735 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. In addition to the control condition (CTRL), the supplement of selected concentrations of PGRs was tested: 0.5 mg/L BAP, 0.5 mg/L *mT*, 0.5 mg/L *mTR*, and 0.5 mg/L *oTR*. The concentration of 0.5 mg/L was chosen because it yields the most efficient shoot multiplication according to many authors (Brugaletta et al. 2009; Osório et al. 2012; El Bouzdoudi et al. 2017; Cavallaro et al. 2017; Saïdi et al. 2019). Ten shoot explants (approximately 2 cm) per bioreactor have been used. The explants were cultivated following the instructions of the bioreactor manufacturer (<https://www.vitropic.fr>). Shoots were immersed in liquid medium (200 mL) for 2 min every 6 h using an aerating pump.

Shoot cultivation with the different PGRs on SCM and TIS RITA® was repeated thrice.

Shoots utilized for the multiplication test were separated from 60-day-old developed seedlings (derived from the first experiment), and their initial fresh weight and length were 50 mg and 1.3 cm, respectively.

In both cultivation systems, the pH was 5.8, the temperature was constant at 23 ± 2 °C, and the light was with a 16 h photoperiod ($60\text{--}90 \mu\text{mol m}^{-2} \text{s}^{-1}$ flux, cool white fluorescence light). All media were autoclaved at 121 °C for 16 min.

The number of shoots was determined by dividing the number of shoots at the end of each experiment by the initial number of explants cultured in the box and bioreactor. In addition, shoot length was also calculated together with the fresh weight. The number of shoots per explant, shoot length, and shoot fresh weight were recorded after 45 days of culture.

Statistical analysis and figure manipulation

Statistical analysis and graphic rendering of the result were produced using R Core Team, R Studio Team software and ImageJ. The statistical analysis by two-way analysis of variance (ANOVA) with a post hoc test using Tukey's least significant difference ($p < 0.001$) was applied. Images were assembled using Adobe Photoshop CS6.

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Author contributions PC: investigation, formal analysis, methodology, and writing. FB: formal analysis, review and editing, visualization. CA: review and editing. FL: investigation. IDP: investigation. MSL: review and editing. JN: review and editing. GPDS: conceptualization, methodology, writing, review and editing. All authors have read and agreed to the published version of the manuscript.

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Data availability Data generated or analyzed in this study are included in the manuscript and in the supplementary materials.

Declarations

Ethical approval Not applicable.

Conflict of interest The authors declare no conflict of interest.

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