Establishment of In Vitro Cultures and Investigation of Micropropagation Possibilities In Shoot Tip Explants of Different Mastic Tree (Pistacia lentiscus L.) Genotypes

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Abstract

The aim of this study was to perform in vitro clonal micropropagation of mature mastic trees (*Pistacia lentiscus* L.). In addition, browning problems in culture media, different sampling periods and success of genotypes were investigated. In the study, 1–2-years-old wood cuttings which are approximately 20–25 cm belonging to 4 different genotypes were used as the starting plant material. Samples were taken in November, December and January. The shoot tips obtained by forcing the hardwood cuttings in distilled water were used as explants in tissue culture. When all genotypes are averaged, the average shoot value per cutting was found at the highest level in January with 6.57. The application of paraffin, which covers the basal parts of the explants, significantly reduced or even completely prevented browning. In the second stage of the January period, 51.33% of the explants formed adventitious buds in MS media containing 1 mg/l BAP (6-benzylaminopurine) and 0,5 mg/l GA_3 (gibberellic acid). Explants of wild genotype showed 61.54% rooting in MS medium containing 2 mg/l IBA (indole-3-butyric acid). The rooting success achieved in the wild genotype indicates that it is possible to micrograft shoot tips of culture clones onto rooted wild genotype explants rather than related species. If it was further developed, this method would stand out as the most reasonable method for commercial micropropagation of mastic trees.

Key Message

Pistacia species have really problem in plant tissue culture because of the browning problem. We tried to solve this problem in this research. We were able to root especially in a *P. lentiscus* genotype.

Introduction

Pistacia lentiscus L. (mastic tree) is an evergreen species belonging to the Pistacia genus of the Anacardiaceae family spreads all over the Mediterranean coasts and is an important part of the maquis vegetation (Al-Saghir and Porter 2012). Chia variety (*Pistacia lentiscus* L. var. *chia* Duham.) of this species has been cultivated for its valuable resin and is being cultivated economically with only male individuals. There are serious differences in resin yield, taste and aroma between wild and cultivated mastics.

The production of mastic gum is based on releasing the resin by making incisions on the trunk and thick branches in the period between July and September. The most important ecological factor limiting the cultivation of the mastic tree is frost. Mature trees are usually cared without irrigation. It is a slow-growing plant so harvest starts with small amounts, 6–7 years after planting (Pericos 1993). Except for a small amount of production in Çeşme, Turkiye; mastic production is only done in Chios, Greece. Mastic gum is a product that creates high added value and is used in food, medicine, cosmetics, landscaping and industrial areas.

Mastic trees are traditionally propagated by hardwood cuttings, air layering and semi-wood cuttings but problems such as the possibility of application in a narrow period during the year, low rooting rates decrease effectiveness of these methods. In order to establishing new plantations with true-to-type clones economically, necessitate an affordable clonal sapling production as a priority in mastic tree cultivation.

Although progress has been made in micropropagation studies on the mastic tree, no significant success has been achieved up to the present. It can be said that the main reason for recalcitrancy of the mastic tree under in vitro culture conditions is the phenolic compounds secreted from the explants and browning mechanism. This mechanism is explained through the following three phenomena: accumulation of phenolic substances, activity of oxidative enzymes and oxidative stress.

The only record of rooted explants of mature mastic trees under in vitro was reported by Mascarello et al (2007). In this study, where the adaptation rate to the external environment is low, intermodal explants taken from young, wild individuals were rooted in medium containing 0.5 mg/l NAA (naphthalene acetic acid).

Although it couldn't be rooted, more successful results were obtained in terms of shoot development in green tissues obtained by forcing wood cuttings. With the application of 10% NaOCl for 10 minutes on the green shoot tips, 90% of the explants remained alive, while browning was not observed. The maximum shoot number and length were reached in MS medium containing Gamborg B5 vitamins, 0.5 mg/l GA3, and 1 mg/l BAP (Onay et al. 2014; Çalar et al. 2016).

The concentration and composition of phenolic compounds in explants can vary in different genotypes of the same species or at different sampling periods (Das and Srivastav 2015; Baydar 2006). In this study, experiments were performed on browning phenomenon (or phenolic oxidation) by using different genotypes and sampling periods with the aim of investigate micropropagation possibilities of the mastic tree.

Material And Methods

In this study, 20–25 cm wood cuttings were used as the starting plant material. Green shoot tips obtained by forcing the cuttings in distilled water were used as explants in tissue culture. The cuttings were collected from 4 male individuals, aged approximately 20 years, from the Çeşme region of İzmir, Turkiye. These genotypes will refer to as G1, G2, G3 and G4 (wild) in the study and ranking is based on estimated agronomic performance.

Cuttings were collected from all four sides of the trees, 10 per month, between the 19th and 21st days of November and December 2020 and January 2021. Therefore, the research started with 40 cuttings each month and a total of 120 cuttings were used. They were transported to the laboratory in frozen food bags containing ice bags and moist perlite, and were processed within 24 hours at the latest. They were washed first under tap water and then with distilled water. The wounds, especially the bottom parts of the cuttings, were wiped with cotton treated with 70% ethyl alcohol. Then, in the light of the findings of Çalar

et al (2016), they were taken into jars, in groups of five, filled with 250 ml distilled water and forced to form new shoots. The distilled water in the jars was renewed once a week and 0.5 cm of the bottom of the cuttings was cut with pruning shears before each renewal. During this time, the cuttings were stored in the climate chamber under 25°C temperature and 16 h light photoperiod conditions.

MS nutrient medium (Duchefa Biochemie, Catalog No: M0222) was used as the basic nutrient medium in the study. The 4 different nutrient media used in the study are derivatives of this nutrient medium (Table 1). Two different nutrient media were used in the initial experiments set up to inhibit browning. Uniform nutrient medium was used in the secondary stage subcultures established in order to encourage shoot growth and growth in the rooting media thereafter. These preferences have been shaped by the findings of previous studies on the subject. pH (5.8) and agar (6 g/l) values were kept constant in all media. Approximately 30 ml of medium was filled into each magenta vessel.

Table 1
The content and derivatives of the MS medium used in the experiments.

Chemical Substance	mg/l	Nu / Stage	Content
KNO_3	1900	1 (Control, I)	MS + 30 g/l sucrose
$CaCl_2$	332,02	2 (I)	½ MS + 10 g/l sucrose + 1 g/l A.C.[1]
NH_4NO_3	1650	3 (II)	MS + 1 mg/l BAP + 0,5 mg/l GA_3
$MgSO_4$	180,54	4 (III)	MS + 2 mg/l IBA
KH_2PO_4	170		
FeNaEDTA	36,7		
H_3BO_3	6,2		
$MnSO_4.H_2O$	16,9		
$ZnSO_4.7H_2O$	8,6		
KI	0,83		
$Na_2MoO_4.2H_2O$	0,25		
$CuSO_4.5H_2O$	0,025		
$CoCl_2.6H_2O$	0,025		
Glisin	2,0		
myo-İnositol	100		
Nikotinik asit	0,5		
Piridoksin-HCl	0,5		
Tiyamin HCl	0,1		
[1] Activated Carbon			

Initial experiments could be done according to the number of green shoots obtained from the cuttings and a certain priority order was followed between the applications. The shoot tips separated from the cuttings were immediately put in water and washed under tap water. Surface sterilization of the explants was achieved with a 15% commercial bleach (sodium hypochlorite) solution diluted with distilled water for 10 minutes and rinsed with sterile distilled water 3 times for five minutes. Shaking processes were done manually and after rinsing, the explants were allowed to dry a little on filter papers. Evaluations after sterilization and applications were made on the 14th day after planting. Explants were planted in

magenta vessels three each as 15–25 mm. Care was taken to create as few wounds as possible on the explants during planting. Magenta vessels, whose planting were completed, were stored under 25^{o}C temperature and 16 hours light photoperiod conditions.

In the first stage, plant growth regulators were not used in the nutrient media in order to prevent or reduce the darkening of the cultured explants, and 5 different applications were planned, except for the control group. These include; i) planting the explants in half strength MS (1/2 MS) media containing a low amount (10 g/l) of sucrose and activated carbon (1 g/l) (A), ii) coating the cut explant bottoms with paraffin (P), iii) keeping the planted magentas in a refrigerator at 4°C cold and dark conditions for 24 hours before being taken to the climate chamber (S), iv) using 1 g/l fungicide (50% Captan WP) instead of sodium hypochlorite (NaOCl) in the surface sterilization of explants (30 min) (F), v) Treating the explants with 50 mg/l gibberellic acid solution during 60 min (G). These applications are indicated by the abbreviations K (control), A, P, S, F, and G in the tables. Simple MS medium (containing 30 g/l sucrose and 6 grams agar) was used as the control group. Each application was carried out with 15 explants in 5 replications.

There are exemplary studies showing that activated carbon used in nutrient media, lower mineral salt or sugar concentrations can reduce or affect the browning encountered in various species (Patel et al. 2018; Boudabous et al. 2010; Mereti et al. 2002; Rana et al. 2016; Altunok ve Er 2013). It is thought that activated carbon also inhibits the activity of oxidative enzymes by creating a dark environment, but it did not show the expected effect (Patel et al. 2018).

Since browning is an oxidative reaction, materials such as paraffin can be used to cut off the contact of cut surfaces with oxygen and create a physical barrier against phenolic substance accumulation. In a study conducted on *Dioscorea alata*, the browning rate of 84% was reduced to 18% with this method and 90% viability was achieved in explants (Bhat and Chandel 1991).

Dobranszki and Teixeira da Silva (2010) stated that oxidative enzymes are affected by environmental conditions and that increased oxidative enzyme activity under the presence of light and high temperature conditions cause more intense browning. Solutions for reducing the activity of oxidative enzymes such as PPO (polyphenol oxidase) are used by keeping the culture media in dark or low temperature conditions for the first few days. The most common approach to browning problems also in the food industry is low temperature applications (Ohlsson 1994).

It has been reported in various publications that as the concentration and application times of sterilant substances such as ethanol, Tween-20, sodium hypochlorite (NaOCl), hydrogen peroxide (H2O2), mercury chloride (HgCl2) used in the surface sterilization of explants increase, the browning intensifies (Nayanakantha et al. 2010; Alp et al. 2010; Moradpour et al. 2016; Ersalı et al. 2017; Salam et al. 2019). It has been stated that these sterilants generally have a toxic effect, preventing growth and development in vitro or causing browning (Nartop 2018).

In a study investigating the interaction of gibberellic acid with phenylalanine ammonium lyase (PAL) enzyme, it was reported that shaking Phalaenopsis explants in 25 mg/l GA3 solution for 20 minutes significantly reduced the browninging and it was suggested that gibberellic acid might inhibit the activity of oxidative enzymes (Zhou et al. 2009).

Synthetic, colorless paraffin, sterilized in the autoclave, was melted in a heated magnetic stirrer in the biosafety cabinet before use and applied warm. The severity of blackening or browning in explants was assessed at day 14 from planting in four grades (0: no substance release or browning, 1: low-intensity browning including superficial darkening of the cut parts, 2: medium intensity browning, 3: intense, complete darkening). The observations were then compared using the formula outlined by Yang et al (2010): Σ (dark intensity × number of explants at corresponding darkening intensity) / (total number of explants).

Explants that remained healthy (uninfected, had low or no browning, had sufficient green tissue, not heavily affected by chlorosis and shoot tip necrosis) in the PGR-free starting media were subcultured. In the first subcultures (stage II), MS medium containing 1 mg/l BAP and 0.5 mg/l GA3 was used to promote shoot growth, lengthening and shoot formation. The darkened bottoms (1–2 mm) of the explants were removed before subculturing.

In the third stage, MS medium containing 2 mg/l IBA was used to root the explants. Rooting media was renewed once after 3 weeks and evaluated for a total of 6 weeks. During the subculturing process, the explants were moved with precision and no cuts were made at the bottoms. Only undesired callus formations on the stem tip were cleaned.

In the study; the effect of cutting period and genotype on obtaining green shoot tips (obtained explant number / number of sprouted cuttings), the effect of the initial applications to stop explant browning, the effect of the initial applications to shoot development, the effect of cutting period and genotype on browning, the effect of cutting period and genotype on shoot development and adventitious bud (shoot) formation (% of explant forming shoot elongation, % of explant forming bud, number of buds per explant) were investigated.

Initial experiments were set up in a randomized plot design with 5 recurrence so 15 explants totally. IBM SPSS 26 package program was used in the evaluation of the data obtained from the research. The graphics were created by using Microsoft Excel Program.

Results & Discussion

The Effect of Cutting Period and Genotype on Obtaining Green Shoot Tips

It was determined that the cuttings formed new shoots within an average of 14 days after they were placed in water (Fig. 1). The degree of pollution of the media by the substances released from cuttings of

different genotypes varied. The genotype number 4 (wild individual) was formed shoots at most and in the shortest time for valid for all 3 periods. On the other hand, genotype 3 formed new shoots in the least number and the longest time. The average number of shoots (except those showing abnormal growth, in plantable height and health) obtained from the cuttings is given comparatively in Fig. 2. An average of at least 2.38 and at most 9.2 shoots per cutting occurred. In terms of the sampling periods, the average number of shoots formed by the cuttings belonging to the 1, 2 and 3 genotypes gradually increased, while the opposite situation was observed in the 4 genotype. When the average of all genotypes is taken, the average shoot value per cutting was found at the highest level in January with 6.57.

With its long internodes and sparse branching, genotype 3 produced the least amount of shoots from the cuttings. It is seen that inferences can be made about the sprouting capacity based on the characteristics and regeneration capabilities of different genotypes in the natural environment. Sprouted cutting rate of 82.5% obtained in this study was higher than the average value (40–50%) stated by Çalar et al (2016).

Infection and Contamination Rates in Explants

After sterilization procedures, infection was encountered at a rate of at least 1.67% and at most 40.00%. The findings of the genotype and sampling periods are given in Table 2. While the average infected explant rate was 6.11% in December, it was 21.33% and 21.54% in November and January. While the lowest mean infection value was detected in genotype 3 with 6.67%, genotype 2 with a rate of 27.41% appeared as the highest mean infection value. In the sterilization performed with fungicide (captan active ingredient) (not specified in the table), infection rate was 73.32%. No contamination was observed in the ongoing subcultures of the study.

Cuttings were collected in rainy weathers in November and January, and in windy, dry weather in December. The difference in infection rates may be related with exposure of the cuttings to moisture. The form and branching frequency of the donor plants seem to have affected the infection rate. While genotype 2 has a compact form, genotype 3 shows very sparse branching and leafing. Therefore, receiving more sunlight and higher aeration capacity of genotype 3 may have been reflected in the infection rate.

Table 2
Infection rates (%) observed in in vitro.

	November	December	January	Mean (genotype)
G1	40,00% (15)	6,67% (45)	21,67% (60)	18,33%
G2	31,10% (45)	11,10% (45)	40,00% (45)	27,41%
G3	13,32% (15)	6,67% (30)	3,32% (30)	6,67%
G4	13,32% (75)	1,67% (60)	16,67% (60)	10,77%
Mean (period)	21,33%	6,11%	21,54%	
The numbers in parentheses indicate the average of how many explants the ratios given are.				

The Effect of Browning Inhibitory Applications and Period & Genotype of Cuttings on Browning

Of the 540 explants cultured, 362 (67.04%) showed low, moderate or high-intensity darkening (Fig. 3). Browning occurred mostly at low intensity and on the cut bottoms of the explants. This result does not quite coincide with the browning rates of 26.67% in the result of 15% NaOCl in 20 min or 0.00% in the result of 10% NaOCl in 10 min, shared by Onay et al. (2014) and Çalar et al. (2016). It is necessary to add to this the evaluation of the browning intensities seen in the explants. In this study, browning phenomenon were interpreted not as present/absent, but over a four-stage rating and were not ignored, albeit at low severity. By means of the formula explained in the methods, the degree of browning was calculated and it was observed that browning occurred with a minimum of 0.07 and a maximum of 1.73. The browning intensities of the sampling periods, genotypes and applications are given in Table 3.

Table 3
Browning intensity values observed in initial experiments (stage I)

		K[1]	A[2]	P[3]	S[4]	F[5]	G[6]
November	G1	1,22					
	G2	1,13	1,00	0,18			
	G3	1,31					
	G4	1,07	1,00	0,15	1,23	1,00	1,73
December	G1	1,14	1,07	0,21			
	G2	1,28	1,67	0,29			
	G3	1,07	1,08				
	G4	1,00	1,47	0,07	1,07		x
January	G1	1,00	1,00	0,18	1,00		
	G2	1,22	1,13	0,30			
	G3	1,07	1,00				
	G4	1,00	1,00	0,08	1,00		
Values between 0.00–3.00 are shown.							
[1] MS (30 g/l sucrose)							
[2] Half strength (1/2) MS (10 g/l sucrose, 1 g/l activated carbon)							
[3] Paraffin							
[4] Cold, dark stogare for 24 hours							

[5] Fungucide (%50 captan WP, 30 min) sterilization instead NaOCl

[6] Giberellic acid solution (50 mg/l, 60 min)

When the browning values were evaluated over the control groups, it was observed that they were generally in a decreasing or stable course towards January. It was observed that the paraffin application, which was used to prevent phenolic substance leakage and therefore browning by coating the cut bottom parts of the explants, almost completely prevented the browning (Fig. 4). However, blackening was observed again, albeit at a low intensity, with the removal of paraffin after subculture. The use of media with activated carbon and 24-hour cold-dark storage after planting did not show the expected effect.

In the initial cultures (stage I), severe physiological disorders occurred as a major problem in the apical buds rather than the browning and phenolic substance leaks from the basal parts and it limited the number of subcultured plants. First, color changes (from yellow to orange and brown) and then shedding were seen on the leaflets and apical buds (Fig. 5). Shoot tip necrosis is a common physiological disorder encountered in *in vitro* cultures of woody plant species (Surakshitha et al. 2019). Oxidative stress, cytokinins, sensitivity to a specific nutrient (especially inactive ones such as iron, calcium, boron) or mineral salt, absence of the root system, hyperhydration, limited gas exchange in the environment and ethylene accumulation may be the causes of chlorosis and necrosis seen in shoot tips. In particular, oxidative stress has an important and triggering role as it has a cause or effect relationship with other problems (Cassells and Curry 2001). Shoot tip necrosis is characterized as a complex problem because different types of media components or conditions have contradictory results (Bairu et al. 2009). In this regard, even the situation of the donor plants seems worth examining because the young trees in the garden used in this study have a chlorosis that affects almost all the leaves. In *Trichosanthes dioica*, shoot tip necrosis was found to be due to calcium deficiency and was eliminated by supplementation of calcium chloride in the medium (Kishore et al. 2015). There are other studies reporting that shoot tip necrosis encountered in culture media is controlled with increased calcium or boron concentrations (Bairu et al. 2009; Surakshitha et al. 2019). In *Vaccinium* species, it was found that chlorosis was related with iron (Fe) element source and resolved with the use of FeEDDHA as an alternative or together with FeEDTA (Shibli et al. 1997). Donnini et al. (2008) attributed the chlorosis problem encountered in quince (*Cydonia oblonga* Mill.) and pear (*Pyrus communis* L.) to the iron defficiency due to the lack of root system of the explants. In a study conducted on shoot tips of the Red Globe (grape) cultivar, the rate of necrotic shoot tips was 30.44% in half-strength MS medium containing 1 mg/l BAP, while it was over 65.00% in full-strength MS medium containing 3 mg/l BAP (Surakshitha et al. 2019). It has been reported that increasing doses of 2iP (2-isopentenyl adenine) and kinetin (values between 1 and 15 mg/l) in *Cercis canadensis* (Eastern Redbud) cause progressive yellowing and necrosis of shoots and leaves (Mackay et al. 1995). Onay (2016) reported that the presence of BAP in pistachios affects the necrosis of shoot tips, shoot tip necrosis is less in media without cytokinin, but there is no significant difference between the effects of different BAP concentrations (1–8 mg/l). In another study conducted on pistachios, it was observed that chlorosis in leaves and shoot tip necrosis decreased in a medium containing 1/3 less calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) compared to MS medium. Increasing calcium chloride concentration damaged the shoot growth and exacerbated chlorosis. The problem was solved by reducing the subculturing interval from 4–6 weeks to 3 weeks (Dolcet Sanjuan and Claveria 1995).

In this study, it was thought that necrosis and sheddings encountered in mastic tree explants may be closely related to the absence of cytokinin (BAP) in distilled water cuttings or in cultures started *in vitro*. The chlorosis and shedding of the shoot tips in the initial applications of paraffin where blackening is almost never seen supports this possibility. In the preliminary experiments using cytokinin (1 g/l BAP) in the media (the findings are not included here), intense chlorosis and shoot tip necrosis were not observed, while a much more severe medium and explant browning spreading from the bottom parts were encountered. Mascarello et al (2007) stated that high BAP levels may have effects on hyperhydration,

browning and reducing shoot length. From a different perspective, it can be said that the problem is result of interaction between oxidative stress and cytokinin. Accordingly, in the absence of BAP, the effect of oxidative stress occurs intensely on the shoot tips. Kishore et al (2015) reported that the presence of low cytokinin in the medium combined with the absence of roots where endogenous cytokinin biosynthesis takes place, exacerbates shoot tip necrosis. Perez Tornero and Burgos (2000) relieved apical necrosis by dipping apricot shoot tips into BAP solution before planting. Therefore, the use of appropriate low doses of BAP in cultures may make a difference in terms of the balance of browning and shoot growth, which is estimated to be in an antagonistic interaction.

According to the results of the two-way ANOVA test, the effect of the sampling period and genotype on the browning was not found to be statistically significant. Variance analysis test results are presented in Table 4. The average of the browning intensity of each magenta vessel over the control groups was used in the statistical analyzes where the browning was evaluated.

Table 4
Analysis of variance test results on the effect of period and genotype on browning.

Variation Source	Type III Sum of Squares	Degree of Freedom	Mean Square	F	Significance
Corrected Model	0,058[a]	11	0,005	0,810	0,630
Intercept	7,731	1	7,731	1195,030	< 0,001
Period	0,023	2	0,012	1,805	0,176
Genotype	0,010	3	0,003	0,521	0,670
Period * Genotype	0,024	6	0,004	0,627	0,708
Error	0,304	47	0,006		
Total	8,146	59			
Corrected Total	0,362	58			

[a] R Squared = 0,159 (Adjusted R Squared = -0,037)

Subcultures (Stage II and III)

A total of 540 explants (165 in November, 180 in December and 195 in January) were cultured. Of these, a total of 179 explants 23 in November, 43 in December, and 113 in January, were subcultured (stage II). Therefore, the viability and subculturing rate of explants increased gradually over periods. Then, a total of 96 explants, 8 in December and 88 in January, were taken into the rooting medium (stage III). When the initial experiments were examined, it was observed that the highest rate of subculture was 48.33% in cold-dark storage application. This was followed by A (42.00%), P (33.33%) and C (26.11%) groups,

respectively. When all periods were examined, it was determined that approximately half of the explants taken into subculture (stage II) belonged to genotype 4 (85 out of 179 so 47.49%). Similar to the success obtained from the wild genotype of the mastic tree, the wild genotypes are more successful than the cultured ones in tissue cultures of some other plant species. There are case studies demonstrating superior regeneration capacities of wild genotypes in oats, tomatoes and potatoes (Lech et al. 1996; Rokka et al. 1996; Kiviharju et al. 1998). The change in the number of explants taken into subculture (stage II) in different periods and genotypes is given in Fig. 6. Detailed numerical data of subculture processes are presented in Table 5.

Table 5
Numbers of explants subcultured.

		K[1]	A[2]	P[3]	S[4]	F[5]	G[6]
November	G1	5					
	G2	2	0	0			
	G3	0					
	G4	2	9	5	0	0	0
December	G1	0	3 : 3	3			
	G2	0	0	0			
	G3	4 : 4	3				
	G4	7	8	6 : 1	9		
January	G1	10 : 10	12 : 12	7 : 7	11 : 8		
	G2	3 : 2	7 : 2	6 : 6			
	G3	6 : 4	12 : 11				
	G4	8 : 5	9 : 3	13 : 10	9 : 8		

Values indicate the number of explants taken into shoot growth media (stage II) and rooting media (stage III).

[1] MS (30 g/l sucrose)

[2] Half strength (1/2) MS (10 g/l sucrose, 1 g/l activated carbon)

[3] Paraffin

[4] Cold, dark stogare for 24 hours

[5] Fungucide (%50 captan WP, 30 min) sterilization instead NaOCl

[6] Giberellic acid solution (50 mg/l, 60 min)

The Effect of Initial Applications, Period and Genotype on Shoot Development & Shoot Formation

In first subcultures of December (stage II), 4 adventitious bud formation (9.3%) and 4 (9.3%) shoot growth (lengthening) were observed in 43 explants (Fig. 7). In the second stage of January, adventitious bud formation was observed in 58 (51.33%) of 113 explants, while there was no lengthening or normal, healthy development in the existing shoots. These bud formations were observed within two weeks in all genotypes, while the percentage of bud-forming explant rates were 75.00%, 43.75%, 72.22% and 20.51%, respectively. Relatively low bud formation rates were observed in explants of initial culture containing activated carbon, low amount of sugar and mineral salts (group A) were noted (Table 6). In addition to phenolic compounds and toxic products, activated carbon can also adsorb auxin, cytokinin and nutrients in the medium (Pan and Van Staden 1998). For example, the number of shoots decreased with the use of activated carbon in Eucalyptus citriodora Hook (Ahuja 1985) and similarly, a decrease in shoot formation was reported in *Pinus strobus* L with the addition of activated carbon to the media containing BAP (Webb et al. 1988). Since no shoot development or formation was detected, the findings of the November period were not included in the table.

It can be argued that the relatively low bud formation rate of genotype 4, which is less affected by physiological disorders affecting the shoot tips compared to other genotypes, indicates the hormone balance within the explant. Doses and combinations of auxins and cytokinins often play a critical role in achieving plant regeneration (Pan and Van Staden 1998). Auxins are synthesized in actively growing parts such as meristematic regions, coleoptile tips, root tips, germinating seeds, apical buds and young leaves. They have the capacity to affect a whole plant with roles such as cell elongation and root formation by being transported through phloem. They also cause apical dominance by suppressing axillary buds (Hopkins and Hüner 2008). Cytokinins, on the other hand, provide adventitious bud formation (Lavakumaran and Seran 2014). Since it has various roles and functions in plant development, any abnormality in the shoot tips negatively affects a series of physiological events. Shoot tip necrosis, which caused 80% death in *Trichosanthes dioica* cultures 5 weeks after the first symptoms, also negatively affected rooting and root development (Kishore et al. 2015). In *Harpagophytum procumbens*, shoot tip necrosis resulted in death of first apical buds and then formation of lateral buds (Bairu et al. 2009). It can be said that, with utilize cytokinin in the second stage of the culture media, mastic tree explants show a tendency towards bud formation and shoot development rather than rooting after damaged shoot tips. Afterwards, the 1 mg/l cytokinin support cut in the third stage of the cultures and the auxin content provided at 2 mg/l concentration may have caused the adventitious buds to become dormant.

Table 6
Shoot elongation and bud formation performances

Period	G.[1]	I.A.[2]	SE%[3] (NSE[4])	BF%[5] (NBF[6])	BPE[7] (FB[8])	T.N.E.[9]
December	G1	A	0,00% (0)	0,00% (0)	0,00 (0)	3
		P	0,00% (0)	0,00% (0)	0,00 (0)	3
	G3	K	0,00% (0)	0,00% (0)	0,00 (0)	4
		A	0,00% (0)	0,00% (0)	0,00 (0)	3
	G4	K	28,60% (2)	14,30% (1)	0,14 (1)	7
		A	0,00% (0)	0,00% (0)	0,00 (0)	8
		P	0,00% (0)	16,70% (1)	0,17 (1)	6
		S	22,20% (2)	22,20% (2)	0,22 (2)	9
January	G1	K	0,00% (0)	90,00% (9)	0,90 (9)	10
		A	0,00% (0)	66,70% (8)	0,67 (8)	12
		P	0,00% (0)	71,40% (5)	1,00 (7)	7
		S	0,00% (0)	72,70% (8)	0,73 (8)	11
	G2	K	0,00% (0)	66,70% (2)	0,67 (2)	3
		A	0,00% (0)	0,00% (0)	0,00 (0)	7
		P	0,00% (0)	83,30% (5)	1,83 (11)	6
	G3	K	0,00% (0)	100% (6)	1,00 (6)	6
		A	0,00% (0)	58,30% (7)	0,58 (7)	12
	G4	K	0,00% (0)	0,00% (0)	0,00 (0)	8
		A	0,00% (0)	0,00% (0)	0,00 (0)	9
		P	0,00% (0)	30,80% (4)	0,31 (4)	13

[1] Genotypes

[2] Initial Applications

[3]% Explant rate showing shoot elongation (Number of explants showing shoot elongation / Total number of explants)

[4] Number of explants showing shoot elongation

[5]% Rate of bud-forming explant (Number of explants forming buds / Total number of explants)

Period	G.[1]	I.A.[2]	SE%[3] (NSE[4])	BF%[5] (NBF[6])	BPE[7] (FB[8])	T.N.E.[9]
		S	0,00% (0)	44,40% (4)	0,44 (4)	9

[1] Genotypes

[2] Initial Applications

[3]% Explant rate showing shoot elongation (Number of explants showing shoot elongation / Total number of explants)

[4] Number of explants showing shoot elongation

[5]% Rate of bud-forming explant (Number of explants forming buds / Total number of explants)

[6] Number of explants forming buds

[7] Number of buds per explant (Number of buds formed / Total number of explants)

[8] Number of buds formed

[9] Total number of explants

It was determined that the buds formed in stages II, did not elongate or remained very short, showing abnormal growth. According to the results of the two-way ANOVA test, it was determined that the effect of the period on the bud formation of the explants was very important ($p < 0.001$). Comparison of bud-forming explant rates are given in Fig. 8. The Duncan multiple comparison test results are given in Table 7 and variance analysis results are given in Table 8.

Table 7
Duncan multiple comparison test results of bud formations.

Period	Bud Formation	Genotype	Bud Formation
November	0.00 b	1	1.58 a
December	0.25 b	2	1.00 a
January	1.41 a	3	1.44 a
		4	0.39 b

Table 8
Variance analysis results of bud formations.

Variation Source	Type III Sum of Squares	Degree of Freedom	Mean Square	F	Significance
Corrected Model	44,117[a]	9	4,902	9,261	< 0,001
Intercept	8,256	1	8,256	15,598	< 0,001
Period	18,018	2	9,009	17,021**	< 0,001
Genotype	2,267	3	0,756	1,428	0,244
Period x Genotype	9,549	4	2,387	4,510	0,003
Error	29,641	56	0,529		
Total	132,000	66			
Corrected Total	73,758	65			
[a] R Squared = 0,598 (Adjusted R Squared = 0,534)					
** indicates significance at level p < 0.001					

The Effect of Cutting Period and Genotype on Rooting and Callus Formation

In the third stage (MS media containing 2 mg/l IBA), 38.64% of the subcultured explants formed callus while no callus was observed in the explants of genotype 3. Calluses, without exception, were developed from the wounds of the shoot tips (Fig. 9). They are of medium hardness, spongy structure and have a brown appearance.

At the end of 3–4 weeks, significant swelling, lignification and hardening were observed in the bottom parts of almost all of the explants taken to the third stage. Rooting was seen only in genotype 4 with a percentage of 61.54% and an average number of roots per rooted explant of 2.69 (Fig. 10). It does not seem possible to infer that the wild genotype is rooted because it secretes less phenolic compounds or shows less browning. In his rooting experiment conducted on 4 different juvenile genotypes grown from seeds germinated in vitro, Kılınç (2013) determined rooting rates ranging from 0.00 to 94.15%, including 25% rooting obtained in an auxin-free medium (only in one genotype). Kostas et al. (2016) obtained rooting rates of 80.0%, 52.5%, 40.0% and 5.0% in cuttings of 4 different culture clones of mastic tree with the application of 4 g/l K-IBA (indole-3-butyric acid potassium salt). This result shows that the genotype factor plays an important role in rooting. According to İsfendiyaroğlu (2003), high IBA doses of 20,000 ppm and above seem necessary for rooting mastic tree cuttings. Considering this, it is predicted that IBA doses above 2 mg/l may be useful for rooting the explants of other genotypes in this study.

Of the 16 explants that formed primary roots, 4 of them with sufficient size and healthy appearance were transferred to pots filled with peat, but their adaptation to the external environment could not be achieved. Explants that did not root in in vitro environments have completely stopped their development or lost their vitality as a result of spreading browning on the tissues.

Findings for the third stage are shown in Table 9. No explants were cultured to this stage during the November period. In the December period, rooting and callus formation were not observed in a small number of explants from 3 different genotypes. Therefore, only the January period data are given in Table 9. The effect of genotype on both callus and root formation was statistically significant ($p < 0.001$). One-way ANOVA test results are presented in Table 10.

Table 9
Callus & root formation percentages of genotypes and Duncan test results.

G[1]	CF%[2] (NFC[3])	Duncan results	RP%[4] (RE[5])	Duncan results	AR[6] (TR[7])	Total Explants
G1	%70,27 (26)	1,86 a	%0,00 (0)	0,00 b	0 (0)	37
G2	%40,00 (4)	1,00 b	%0,00 (0)	0,00 b	0 (0)	10
G3	%0,00 (0)	0,00 c	%0,00 (0)	0,00 b	0 (0)	15
G4	%15,38 (4)	0,40 bc	%61,54 (16)	1,60 a	2,69 (43)	26

[1] Genotypes

[2]% Callus formation (Number of explants forming callus / Total number of explants)

[3] Number of explants forming callus

[4]% Rooting percentage (Number of rooted explants / Total number of explants)

[5] Number of rooted explants

[6] Average number of roots (Total number of roots / Number of rooted explants)

[7] Total number of roots

Table 10
Variance analysis results of callus and root formations.

	Variation Source	Type III Sum of Squares	Degree of Freedom	Mean Square	F	Significance
Callus Formation	Between Groups	19,886	3	6,629	14,089**	< 0,001
	Within Groups	14,114	30	0,470		
	Total	34,000	33			
Rooting	Between Groups	18,071	3	6,024	41,070**	< 0,001
	Within Groups	4,400	30	0,147		
	Total	22,471	33			

** indicates significance at level p < 0.001

Conclusion

There are successful results in several tissue culture studies on mastic trees, especially in studies using seeds and juvenile plants. However, there are some drawbacks in these studies, such as the lack of clonal propagation due to explant selection, the occurrence of somaclonal variations and the inability to determine the sex of the developing plants. Clonal propagation is required to ensure the agronomic performance of these slow-growing trees.

As a result of this study, adventitious bud formation was achieved and rooting was observed in shoot tip explants of mature mastic trees. The rooting gain in the wild genotype indicates that it is possible to micrograft shoot tips of cultured clones onto rooted wild genotype explants. If developed, this method would be the most reasonable method for commercial micropropagation of mastic trees, as the rootstocks will also show homogeneity. January period and genotype 4 revealed prominently the effect and importance of different period and genotype options as predicted. In future studies, it is thought that increase the frequency of subculture periods and the use of low concentrations of cytokinins in the water where the cuttings are kept or initial culture media may be beneficial. It is considered necessary to study the appropriate BAP concentration in terms of browning and growth balance at the shoot tips. It is thought that low-intensity browning of the explants can be ignored if this balance is achieved, that is, in the presence of healthy shoot tips. Thus, there may be no need for various strategies to prevent browning, which is already of low intensity. However, there is a possibility of making a difference if paraffin application is also used in subcultures. Methods such as adding oxidative enzyme (phenylalanine ammonium lyase, polyphenol oxidase, peroxidase) inhibitors to the nutrient medium or using silver nanoparticles (AgNP) that do not harm plant tissues instead of NaOCl in the surface sterilization of

explants are open to testing. In the rooting phase, it would be appropriate to study with different genotypes and higher IBA concentrations. The results are promising for micropropagation of high-yield mastic clones.

Declarations

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Figures

Figure 1

Sprouted buds on the mastic tree cutting

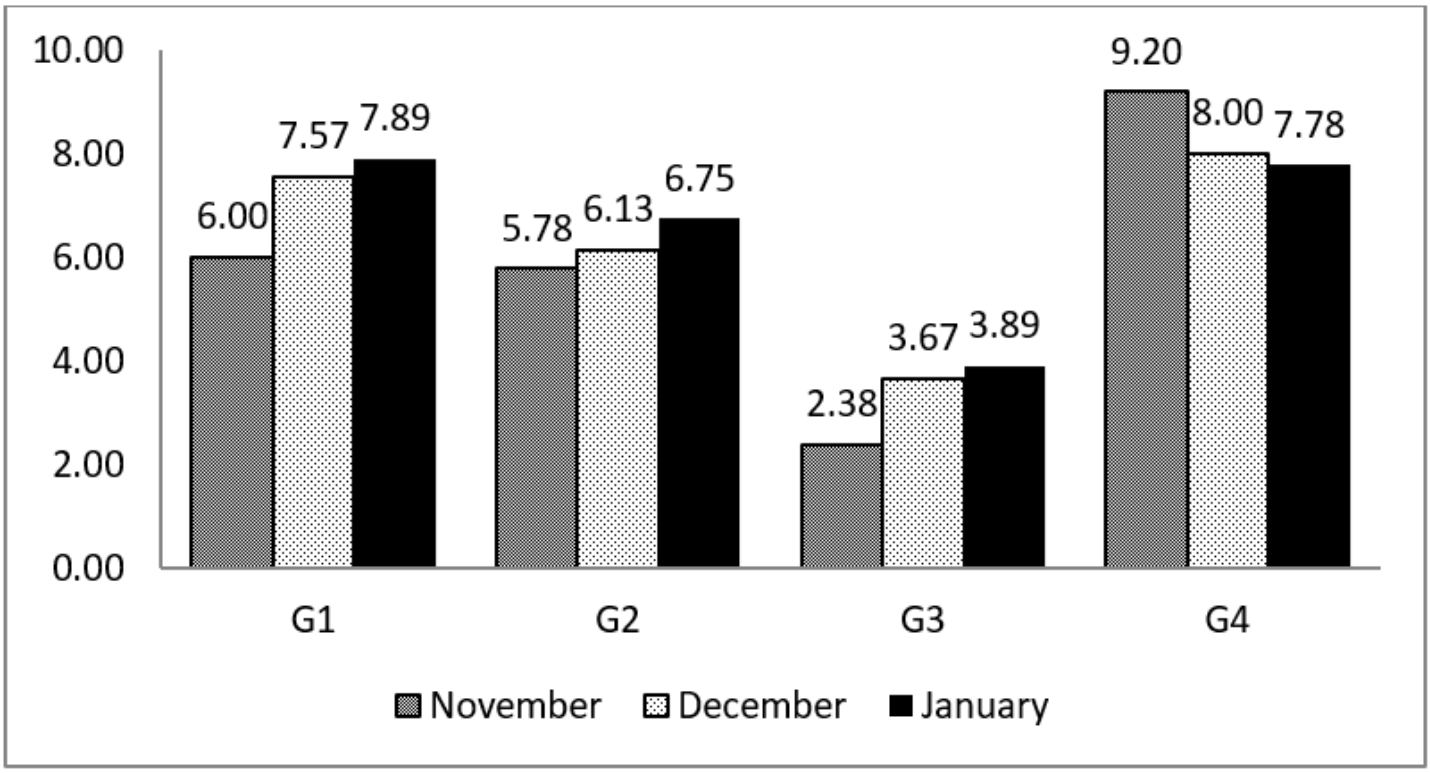

Figure 2

Changes in the number of green shoot tips obtained per sprouted cutting.

Figure 3

Moderate intensity browning on explants

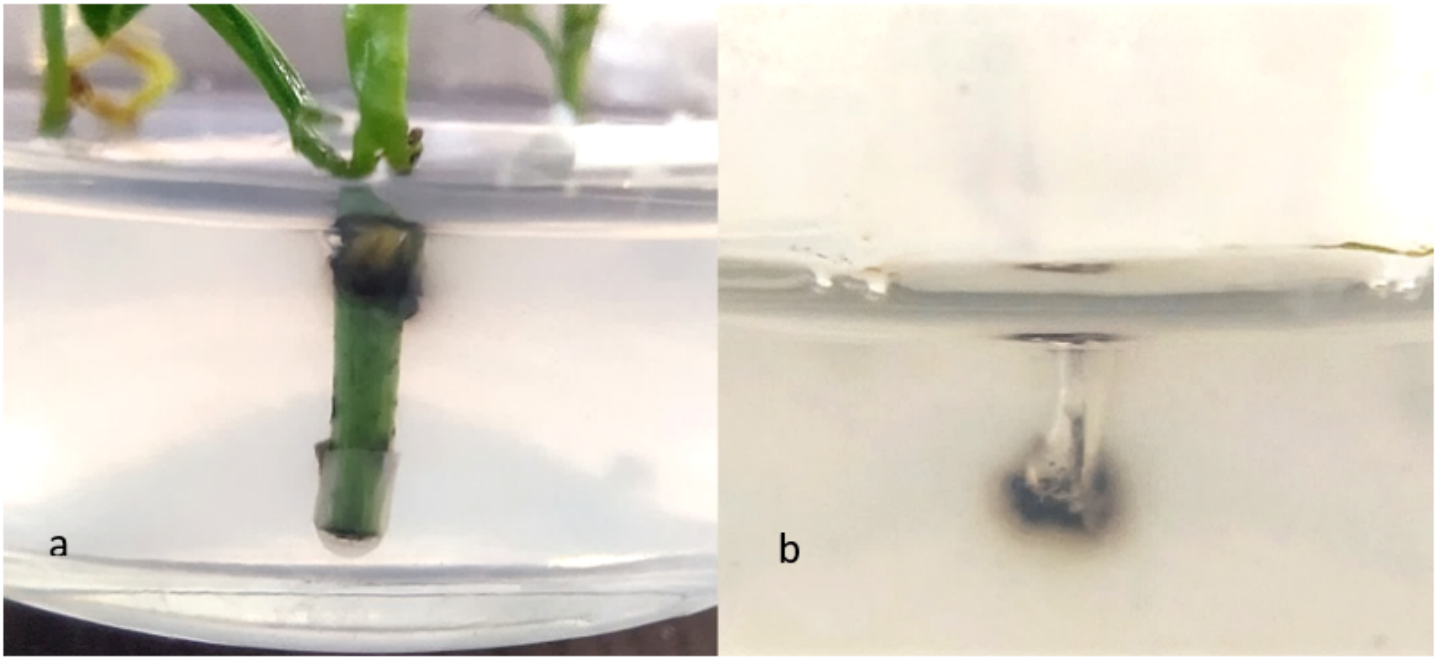

Figure 4

The degree of pollution of the paraffin-treated (a) and untreated (b) explants

Figure 5

Physiological disorders in leaves and apical buds.

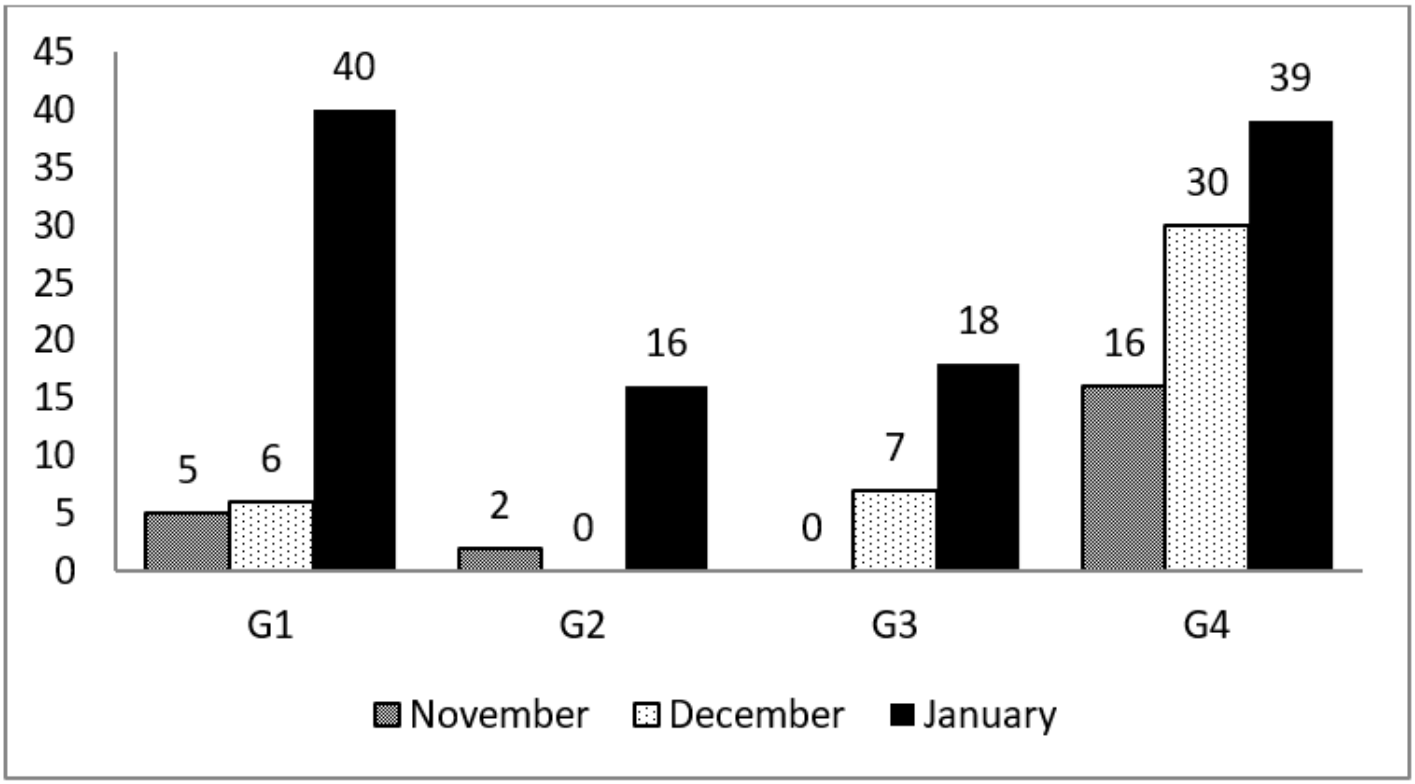

Figure 6

Changes in the number of explants taken into the second stage subcultures in different periods and genotypes (pieces).

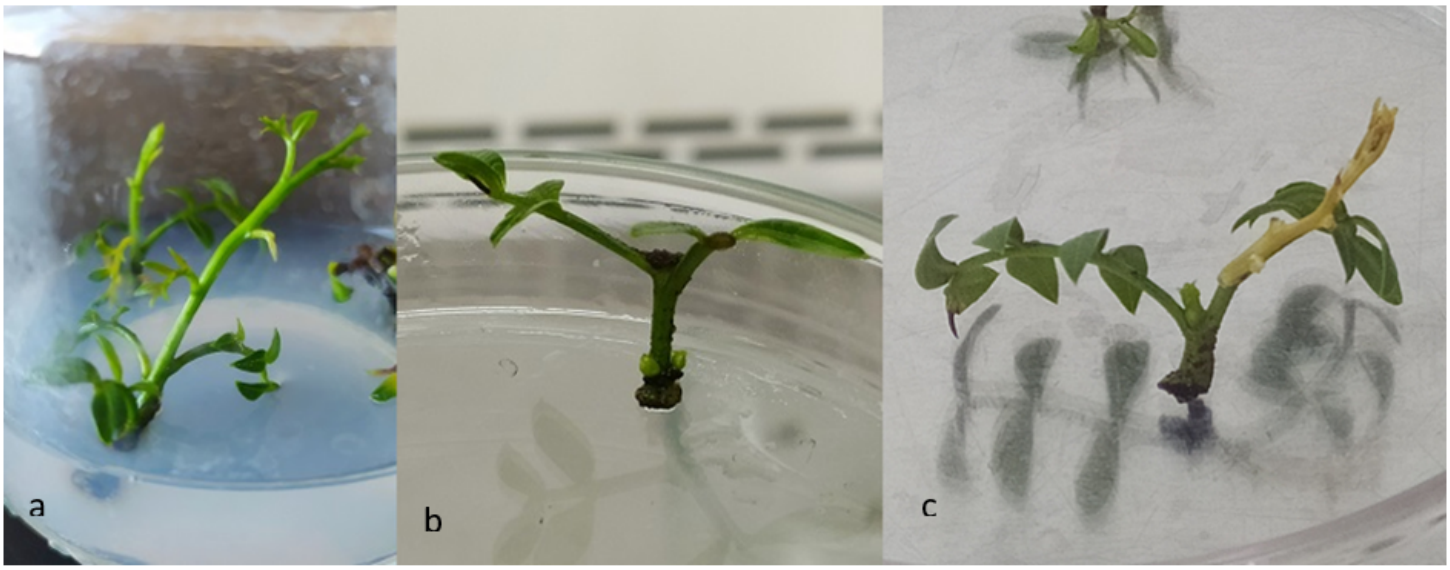

Figure 7

Shoot elongation (a) and bud formation (b and c) observed in explants.

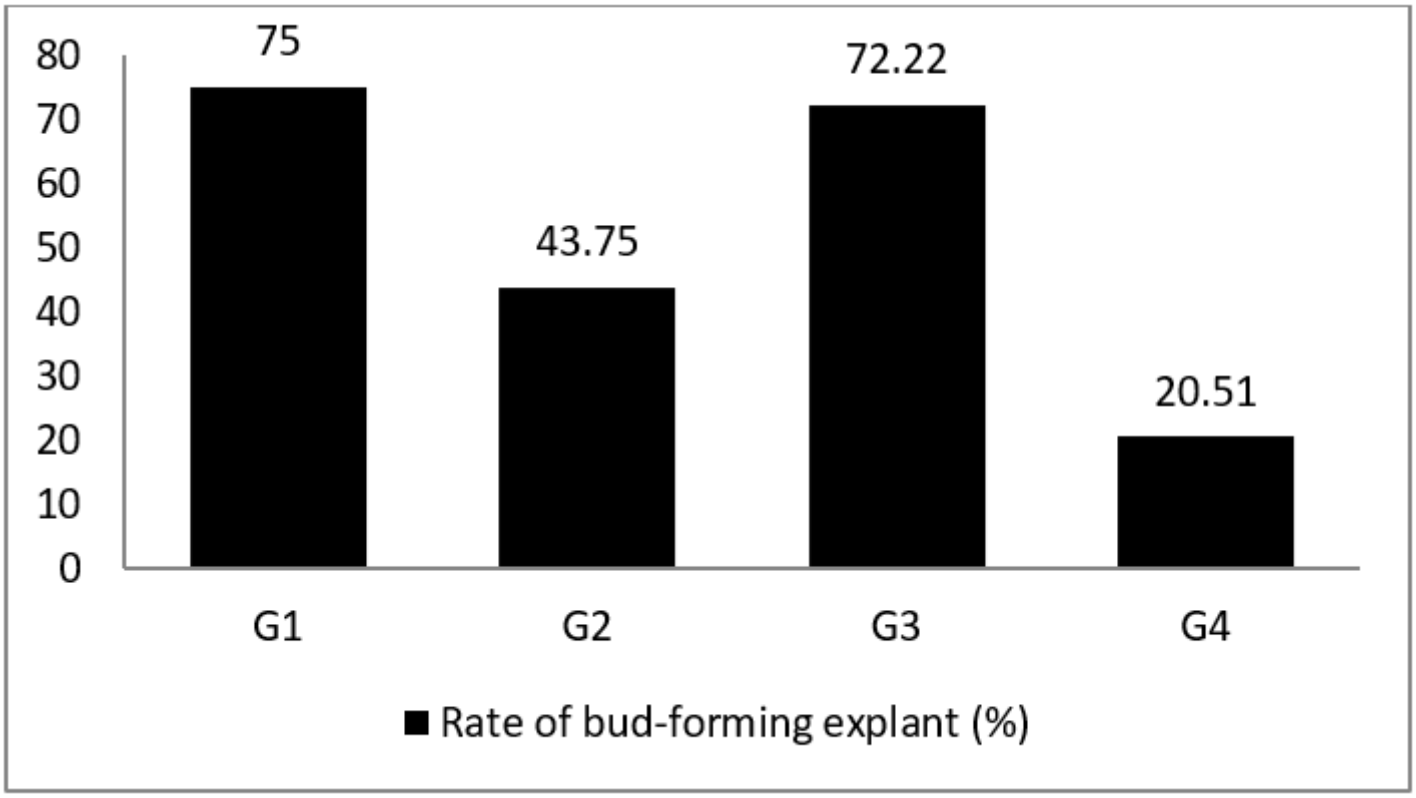

Figure 8

Percentage of bud-forming explant in different 4 genotypes (%, january samples are shown.)

Figure 9

Callus formations

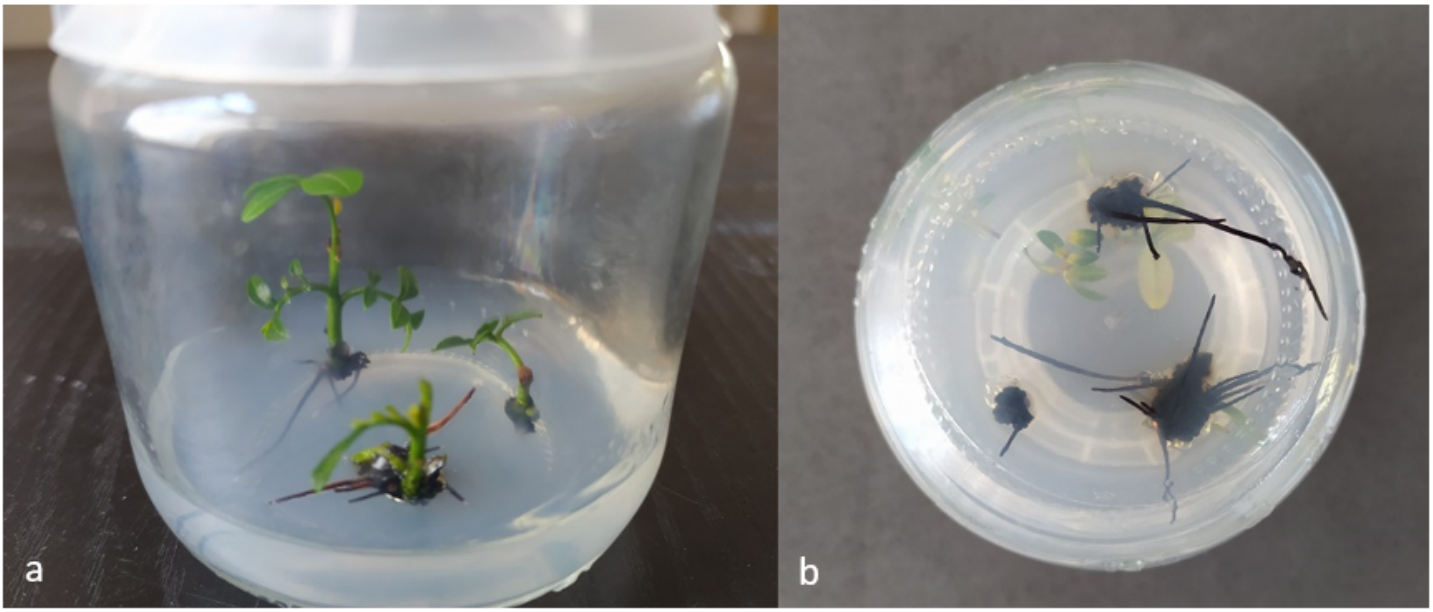

Figure 10

Side (a) and bottom (b) views of rooted mastic tree explants.