Article

In vitro establishment of two cultivars released from strawberries: strawberry and raspberry

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Abstract

The development of protocols for the production of healthy plants *in vitro* is important to satisfy the growing demand for strawberries in northern Mexico as an alternative to traditional fruit trees such as walnut and apple trees. *In vitro* strawberry and raspberry establishment techniques were evaluated from meristematic explants, apices and internodes. A convective disinfection method was used, where disinfecting agents were used as a 70% ethanolic solution, then they were immersed in a 3% commercial solution solution of sodium hypochlorite: water (1:6), for 15 min. Finally, in 0.05% timerosal® solution for 15 min. Apices were evaluated in the case of strawberry apices, and meristems and internodes in the raspberry. For the establishment, Murashige and Skoog salts added with cytokinin 6-benzylaminopurine (BAP), gibberellic acid (GA) and indole butyric acid (AIB) in different concentrations were used for the strawberry case. In raspberry, MS salts added with BAP, thidiazuron (TDZ), GA and AIB were used. The percentages of establishment, necrosis and contamination were evaluated in both cases. The planting of apices in the case of strawberry and its disinfection system, allowed its establishment *in vitro*, the variables were analyzed by nonparametric tests showed that the best treatment was TT1 with 70% effectiveness. For raspberries, the case of internodes was better during establishment than in the case of meristems, and the best treatment was TT4, with BAP + GA without auxin, with 57% effectiveness.

Keywords: berries, 6-benzylaminopurine, meristems, micropropagation, thidiazuron.

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Introduction

The term tissue culture refers to the development of plant cells under *in vitro* conditions, said cells are cultured aseptically in a culture medium of defined chemical composition and incubated under controlled environmental conditions; encompassing a heterogeneous group of techniques to carry out such an end (Roca and Mroginski, 1991; Purohit, 2012). Within these techniques, micropropagation is found, which is widely used in the mass production of high quality genetic material for large scale cultivation, genetic improvement, genetic conservation and research (Jaakola *et al.*, 2001; Gajdošová *et al.*, 2006; Alanagh *et al*., 2014).

Among the benefits of micropropagation are: a single explant can be multiplied thousands of times, the propagation periods are shorter, there is homogeneity in the propagated crops, it allows the production of superior quality plants with resistance capacities and tolerance to biotic or abiotic stress factors, among others (Yesil *et al.*, 2010; Hussain *et al.*, 2012). Micropropagation via meristems or axillary shoots is highly recommended to obtain genetic homogeneity in the mass production of fruit plants, with characteristics identical to the mother plant (Song and Sink, 2004; Ostrolucká *et al*., 2007).

The traditional propagation of the commercial strawberry plant (*Fragaria* x *ananassa Duch*) was carried out through stolons, which is not always adequate due to the vulnerability and susceptibility of these to pathological agents, such as *Botrytis cinerea*, *Fusarium* spp. and *Penicilum* (Clavijo *et al.*, 2010). Therefore, after the propagation and establishment of the crop, large losses occur, up to 50%) of the production, caused by the affectations caused by microorganisms (Guédez *et al.*, 2009; Ying *et al.*, 2009). In 1974, Boxus described a method for the mass micropropagation of strawberry plants by means of the technique of axillary shoots, since then the production of strawberry cultivated *in vitro* has evolved, taking place nowadays in many countries of the world.

These micropropagated plants are used to establish plantations, and it has been found that they show more flowers, higher fruit yield per hectare, more stolons per plant and greater vigor, in comparison with conventionally propagated plants (Tan *et al.*, 2003). In addition, this method has been successful in the prevention of diseases transmitted in soil and plants (Moradi *et al.*, 2011).

On the other hand, the propagation of raspberry *Rubus idaeus* sp.) Is carried out by means of root staking, with which a high propagation percentage and a good quality of plants is achieved. However, it is essential that the quality and age of the mother plants be monitored, since after two or three years the percentage of rootedness decreases considerably, while the risk of diseases and contamination by viruses increases; therefore, this method does not ensure the production of disease free plants. In addition, sometimes the staking method has very low multiplication rates that do not meet the needs of the nursery industry (González *et al.*, 2000; Wu *et al.*, 2009; García *et al.*, 2014). Previously, micropropagation of vegetative raspberry material has been achieved with good results, using nodal segments or meristematic tissue as explants and thus obtaining disease-free plants (Reed, 1990; Minas and Neocleous, 2007; Arencibia *et al.*, 2013).

The objective of this study was to develop methodologies and protocols for the *in vitro* establishment of commercial strawberry berries *cv* Aromas and raspberry *cv* Heritage, using different types of explants in different culture media formulated with plant hormones.

Materials and methods

The test was carried out in the Biotechnology laboratory with materials from the experimental greenhouse of the Faculty of Agrotechnological Sciences of the Autonomous University of Chihuahua, from where strawberry, *cv* Aromas, and raspberry *cv* Heritage, from which the vegetative material used in the present investigation was collected to be established *in vitro*, in 2015.

For the disinfection of the vegetative material in strawberry (*Fragaria* x *annanasa* Duch. *cv* Aromas)

In this case, for the disinfection of strawberry plants apical buds were selected that did not show symptoms of disease. They were cut and stored in sterile distilled water with 150 mgL^{-1} of ascorbic acid prior to handling. After 1 h, the shoots were immersed in aqueous solution of 5% alkaline soap with 2 drops of Tween-80 for 15 min, in constant agitation. Afterwards, they were rinsed under the water tap for 20 min. Under aseptic conditions in a laminar flow hood, the vegetative material was immersed in 70% ethanol for 30 s, then rinsed three times with sterile distilled water.

The explants were then submerged in a commercial sodium hypochlorite solution and distilled water in a ratio of 1:6 for 10 min, shaking gently, and then rinsing again three times with sterile distilled water. Next, the internodes were immersed in 0.05% solution of mercury salts, Thimerosal (Sigma Aldrich®, CAS 54-64-8) for 10 min, then rinsing with sterile distilled water in three occasions.

For *in vitro* establishment from apical strawberry shoots (*Fragaria* x *annanasa Duch*. *cv* Aroma)

The already disinfected apical buds were seeded in 4 different formulations of culture media, three of them with micro and macro elements, added with organic according to the modified MS formulation, see table 1 (Murashige and Skoog, 1962), supplemented with 1.11 µM benzyl aminopurine (BAP), 0.87 µM gibberellic acid (GA) and 2.95 µM indole butyric acid (IBA) for the first control treatment (TT0); the first treatment (TT1) was supplemented with 4.44 µM of BAP, 0.29 µM GA and 4.92 µM of IBA; also, the second treatment (TT2), with 2.22 µM BAP, 0.29 µM GA and 0.49 µM of IBA. Finally, another culture medium formulated with 50% MS salts and vitamins, supplemented with 0.44 µM BAP, 0.29 µM GA and 4.92 µM IBA was identified as the third treatment (TT3), using the suggested growth regulators. by several authors who have reported success in their formulations, such as (Boxus, 1974; de Lopez, 2001; Beltran, 2002; Caboniet *et al*., 2008) (Table 1).

Table 1. Formulation of culture media for strawberry *cv* Aromas, supplemented with cytokinin (benzylaminopurine BAP), auxins (indolbutyric acid AIB) and gibberellins (GA), according to previous propagation reports. TT1 by Caboniet *et al*., 2008; TT2, Beltran, 2002; de Lopez, 2001; TT3 and TT4 Boxus, 1974.

Treatment	Formulation (sales MS)	BAP (µM)	GA (µM)	IBA (µM)
TT1	100%	1.11	0.87	2.95
TT2	100%	4.44	0.29	4.92
TT3	100%	2.22	0.29	0.49
TT4	50%	0.44	0.29	4.92

In addition, 1 g L^{-1} of activated charcoal, 5 g L^{-1} of Phyta-gel (Sigma®) and 30 g L^{-1} of sucrose brand Sigma® CAS 71010-52-1 were added to all treatments. The pH was adjusted to 5.8 in all cases and then sterilized at 1.5 kg cm^2 of pressure at 120 °C for 15 min. After sowing the explants, they were incubated at a temperature of 24 ±2 °C, with a luminosity of 86 µmol m^{-2} s^{-1} supplied by LED lamps model T8 TecnoLite® and a photoperiod of 16/8 h during 15 days. After that time, the ex-plantes that showed growth were transferred in the same formulation, for two more weeks. After this lapse of time, the variables evaluated were percentage of contamination, necrosed plants and established explants.

For the disinfection of vegetative material from nodal explants of raspberry (*Rubus idaeus* L. *cv* Heritage)

For the disinfection of raspberry, healthy mother plants were selected. Stem segments 30 cm in length were sectioned with vegetative axillary buds. These segments were sheltered and placed in moistened plastic bags prior to disinfection. The explants were sectioned leaving a vegetative bud per section, and were immersed in 5% alkaline soap solution with two drops of Tween-80 for 15 min. Then, they were rinsed under the tap for 30 min.

They were then transferred to aseptic conditions in a laminar flow hood by immersing the explants in 70% ethanol for 30 s, then rinsing three times with sterile distilled water. They were then immersed in a solution of mercury salts, Thymerosal (Sigma Aldrich®) at 0.05% for 15 min, then rinse three times with sterile distilled water. Subsequently, the explants were immersed in a solution of commercial sodium hypochlorite with sterile distilled water in relation (1:6) for 10 min, agitating gently, to immediately rinse three times. Finally, the explants were immersed in ascorbic acid solution (150 mgL^{-1}) and stored for 24 h at 4 °C.

For the *in vitro* establishment of the vegetative material in raspberry (*Rubus idaeus cv* Heritage)

Two micropropagation techniques were used to introduce vegetative raspberry material into *in vitro* conditions, nodal segments (internodes) and axillary meristems.

In the case of the internodes, after 24 h, they were sectioned in 2-3 cm explants and planted in four different culture media. The first treatment (TT1) consisted of a modified MS medium, added with 19.75 μM BAP and 0.04 μM of IBA, according to Minas and Neocleus (2007). The second one (TT2) was formulated equal to the previous one, but with the difference that 20.2 μM of Tidiazuron (TDZ) was added instead of BAP, since this cytokinin has been widely used in the propagation of vegetative raspberry tissue (Gajdošová *et al*., 2006; Dai *et al.*, 2006; Cappeletti *et al.*, 2006). The third (TT3) was formulated with MS salts and LS vitamins (Linsmaier and Skoog, 1965), in addition to 4.4 μM of BAP, 0.29 μM of GA and 0.49 μM of IBA (Poothong and Reed, 2015). Finally, the fourth culture medium (TT4) was formulated according to Sigarroa and García (2011), which consisted of an MS medium with modified vitamins plus 4.44 μM of BAP and 1.44 μM of GA, as shown in Table 2 In the case of the meristems, these were extracted with the help of sterile clamps and scalpel and using an optical microscope of the VANGUARD® brand-stethoscope type at 2x magnification.

Table 2. Formulation of culture media supplemented with cytokinins, auxins and gibberellins. According to previous propagation reports for raspberry *cv* Heritage. TT1 Minas and Eoclus, 2007; TT2 Gadjosova *et al*., 2006; TT3 salts by Linsmaier and Skoog, 1965; hormones by Poothong and Reed, 2015; TT4 Sigarroa, 2011.

Treatment	Formulation (Sales MS)	Vitamins	BAP/TDZ (μM)	GA (μM)	IBA (μM)
TT1	100%	Modified MS	19.75 BAP	-	0.04
TT2	100%	Modified MS	20.2 TDZ	-	0.04
TT3	100%	LS	4.4 BAP	0.29	0.49
TT4	100%	Modified MS	4.44 BAP	1.44	-
TT1M	100%	Modified MS	4.44 BAP	1.44	-

The bracts were removed from the axillary buds and the meristematic tissue was seeded in modified MS culture medium added with 4.44 μM BAP and 1.44 μM GA (TT1M), according to Sigarroa and García (2011). In all cases, 1 g L^{-1} of activated carbon, 5 gL^{-1} of Phytagel (Sigma® CAS (71010-52-1) as a gelling agent and 30 g L^{-1} of sucrose, brand SIGMA® (CAS 57-50-1) were added. The pH of the culture was adjusted to 5.7 in the case of internodes and six for meristems (Sigarroa and García, 2011; Poothong and Reed, 2015) and then sterilized to 1.5 kg cm^2 of pressure at 120 °C by 15 min.

After sowing, the vegetative material was transferred to a transfer chamber under controlled temperature conditions of 24 ±2°C under conditions of total darkness. After seven days, they were transferred to brightness conditions of 86 μmol m^{-2} s^{-1} supplied by LED T8 TecnoLite® lamps and a photoperiod of 16/8 h. After 21 days, the percentages of contamination, necrosed plants and established explants were evaluated.

Statistical analysis

In the case of strawberry micropropagation, the qualitative variables evaluated were the percentage of contamination, which evaluates the success of the disinfection treatment, the percentage of necrotic plants, which evaluates the formulation of the establishment medium and the percentage

of explants established (Marulanda *et al*., 2000; Sigarroa and García, 2011), the repetitions were 10 units for each of the four treatments, and these were analyzed using a completely randomized experimental design under homogeneous conditions.

These variables were analyzed by the non-parametric Kruskal-Wallis tests due to the small number of repetitions per treatment with which chi^2 is not valid for proportions test, the Dunn test was applied for the separation of Neave means and Worthington (1988), using the Minitab® statistical package version 17.1.0

In the case of the raspberry, the number of necrotic plants, and the established explants, both of the internodes were evaluated. The repetitions were 65 units per treatment. The data were analyzed using the logistic regression model, using as an independent variable the treatments and the response to necrosis and establishment. All data was processed in the IBM SPSS Statistics 20 software.

Results and discussion

Necrosis

Most plants produce phenolic compounds after passing through stages of stress, such as that produced by contact with disinfectants, so caution must be exercised in their concentration when applying them to plant tissues. This procedure is essential to be able to establish vegetative material under *in vitro* conditions (Rostami and Reza, 2012). These compounds affect the growth and survival of the explants, oxidizing the tissues and leading to necrosis and death.

To avoid this, it is advisable to add antioxidants in the environment, as well as increase the frequency of subcultures (Swartz and Lindstrom, 1986). Other options to avoid the oxidation processes that lead to the darkening of the tissues and their subsequent death are the use of a liquid culture medium, change of the gelling agent, use of activated carbon, addition of ascorbic acid and polyvinyl polypyrrolidone; however, since the problem is complex, a comprehensive solution is required that involves a greater number of variables (Azofeifa, 2009, García *et al*., 2010).

In the present investigation, we sought to counteract the oxidation of tissues by applying activated carbon to the culture medium and placing the explants in dark conditions for a period of time. Activated carbon is distinguished from elemental carbon by its absence of impurities and almost graphitic form, a fine network of pores, an extraordinarily large surface area and a volume that gives it a unique absorption capacity, thus helping to reduce effects of tissue oxidation. In the same way, decreasing light intensity prevents oxidation and necrosis of tissues (Swartz and Lindstrom, 1986; Thomas, 2008). In this trial, in the case of strawberry, the treatments that presented the highest percentage of oxidation and subsequent necrosis were TT3, with 40% and TT4, 70%; on the other hand, TT1 and TT2 only presented 10%; that is, a low percentage of necrotic tissue. In the test for raspberry, the percentage of necrosis in general was high, with an average of 78.1%, despite the addition of activated carbon to the culture medium and having placed the explants under dark conditions for 7 days.

The treatment with the lowest percentage of necrosis was TT1, with 27.69% and according to the Mann-Wittney test, the TT1 of meristems showed a statistically significant difference with respect to the other treatments, with a percentage of high necrosis (Table 3). In this technique millimeter portions of tissues from axillary or apical buds are cultured, each portion consists of a fragment of the meristematic region with or without leaf primordia, therefore, these tiny fragments of tissue are more sensitive to oxidation (García *et al*., 2011; George *et al*., 2008).

Table 3. Frequencies calculated for the response from explant to establishment (0); disinfection (1) and necrosis (2); where it can be seen that TT1 provides the best treatment for the three responses, calculated by analyzing the frequency of the SPSS® IBM v20 statistical package.

Treatment			Frequency	Percent	Valid percent	Cumulative percent
TT1	Valid	0	7	70	70	70
		1	2	20	20	90
		2	1	10	10	100
		Total	10	100	100	
TT2	Valid	0	6	60	60	60
		1	3	30	30	90
		2	1	10	10	100
		Total	10	100	100	
TT3	Valid	0	5	50	50	50
		1	1	10	10	60
		2	4	40	40	100
		Total	10	100	100	
TT4	Valid	0	2	20	20	20
		1	1	10	10	30
		2	7	70	70	100
		Total	10	100	100	

Contamination

The most important process prior to the establishment of the crop is the ideal formulation in the application of disinfectants and antioxidants, as well as the times in which the explants are subjected to the disinfection process. Since *in vitro* conditions some microorganisms such as bacteria and fungi find an optimal environment to develop, an unsuccessful disinfection hinders the progress of propagation, so the application of substances and the use of disinfecting techniques are focused on eliminating said unwanted microorganisms, including mercury salts in percentages of 0.05-0.15%. (Mikropavai-Rošana *et al*., 2009; Yildiz, 2012).

Regarding the evaluation of the contamination variable strawberry, *cv* Aromas, it was found that TT 2 showed a higher percentage of explants with the presence of microorganisms (30%), whereas TT 3 and TT4 only showed 10% of contaminated explants. In general, the disinfection protocol presented acceptable values in treatments TT3 and TT4, as shown in Table 3.

Establishment

In the case of strawberry, *cv* Aromas, the four treatments were statistically different when analyzed by the Kruskal-Wallis and Dunn tests, by comparing the differences between treatments. The treatment that behaved the best was the TT4, with a greater number of explants established successfully (70%). In Figure 1 and Figure 2, it is illustrated that effectively the treatment formulated according to Caboni *et al*. (2008), by using the combination of BAP+GA+IBA, hormonal activity is more efficiently potentiated, obtaining a more efficient vegetative response than in the other cases, with a percentage of 70% of established explants.

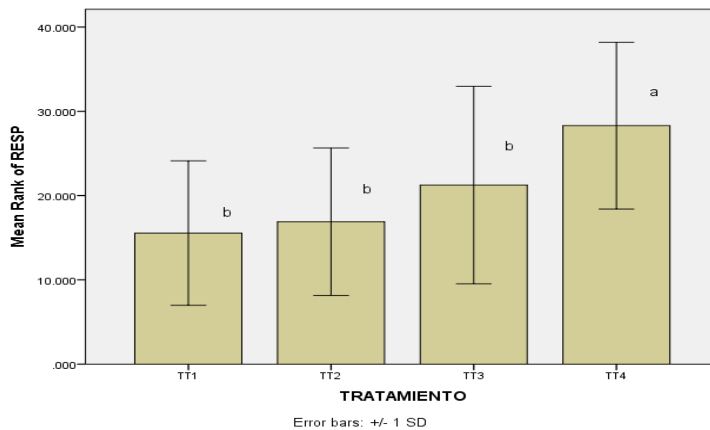

Figure 1. Comparative of the response in establishment, contamination and necroses to the different formulations or treatments evaluated in strawberry, *cv* Aromas. It is appreciated that in the TT4 treatment, the difference is smaller. Nonparametric Kruskal-Wallis tests were used for the separation of means and Dunn's multiple comparisons test, from the SPSS® statistical package version 20.

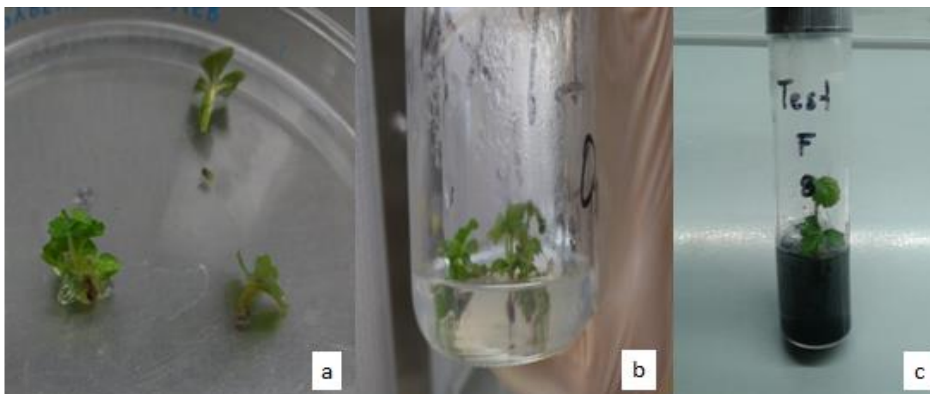

Figure 2. Strawberry vegetative material *cv* Aromas. a. Explants of strawberry apical buds. b. Explants established in culture medium. c. Explante in culture medium with activated carbon.

Raspberry

In the case of raspberry, *cv* Heritage, Kruskal-Wallis and Dunn (reference), to analyze the statistical differences in the use of meristems against internodes, was found to be better internodal tissue to settle, but not in the case of meristems, being that, in this tissue, even though it was the one with the lowest percentage of contamination (1.54%), it had a very low percentage of establishment with respect to intermodal tissue. Figure 3 shows the marked difference between treatments using cuttings against meristem cells.

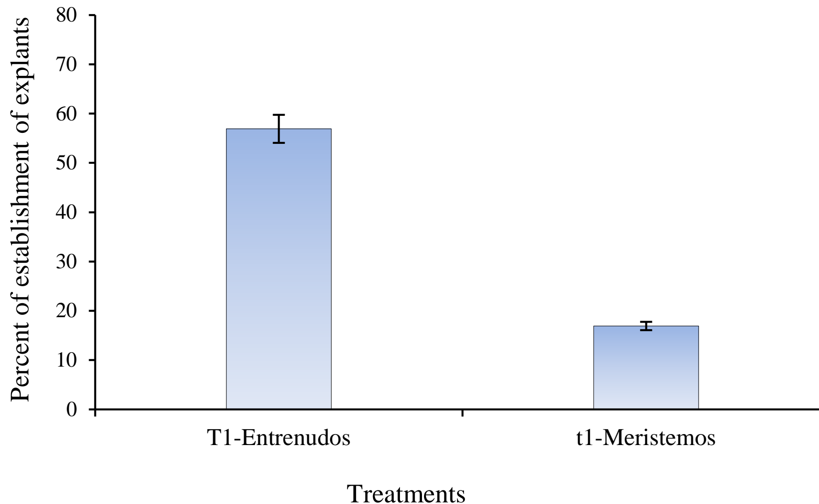

Figure 3. Statistical difference in the establishment of internodes and meristems ex-pressed in percentage, raspberry *cv* Heritage. Using nonparametric Kruskal-Wallis tests and the Mann-Whitney two-proportions test of the Minitab® Statistical Package version 17.1.0.

In the case of the response to treatments with different formulations reported, it was found that the logistic regression analysis, in response to treatments, in the case of the formulation of TT4 (4.44 µM of BAP + 1.44 µM of GA) (Anderson, 1980; Sigarroa, 2011) was the best response to establishment with respect to other treatments, since it had much lower necrosis. It has been reported that the micropropagation through meristems allows not only obtaining high quality plants and more uniform, but also the spread of free specific clones pathogens, achieving reduce endogenous contamination to a minimum level without to apply more aggressive disinfection protocols (Sigarroa and García, 2011). But also, the vegetative response to nutritive formulations, determine to a great extent success, in the prompt response to the culture medium (Sincha *et al.*, 1987; Reed, 1990; Reed *et al*., 2013).

Even though it has been reported that for raspberry, the medium formulated with MS salts, express poor growth and *in vitro* development problems Greenwayet *et al*. (2012), in this case the Figure 4 shows the statistical results of ranges between differences in formulations or treatments evaluated, being effectively TT4 held difference statistically significant at internodes used com vegetative material is establishment and the combination of hormones 44.4 µM of BAP + 1.44 of GA (Sigarroa and García, 2011).

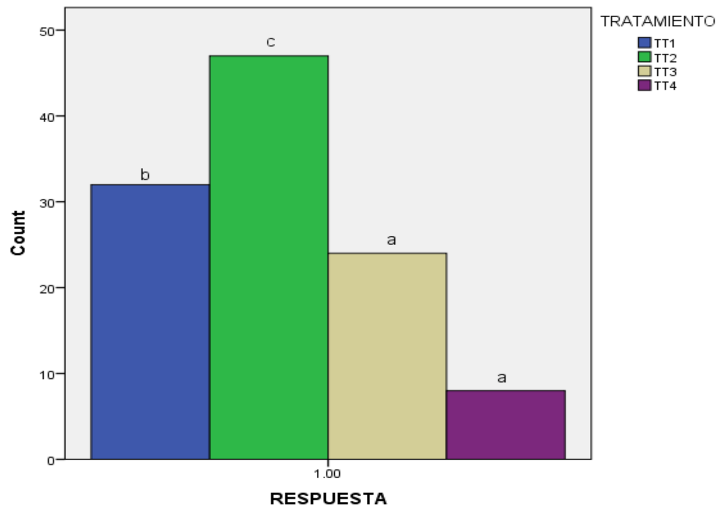

Figure 4. Effect of the formulation on contaminated, necrotic and established raspberry leaflets *cv* Heritage for evaluated treatments. Analyzed using the logistic regression model, using as independent variables the effect of the treatments, to the necrosis by establishment, obtaining X^2. Made in the IBM SPSS Statistics 20 statistical package.

In raspberry only in two treatments it was possible to observe the establishment of the explants. The TT4 of internodes presented the highest percentage of success, reaching 56.92% of established explants (Figure 5). According to the test of two proportions, it was found that there are statistically significant differences between said treatment and the explants of the meristem technique.

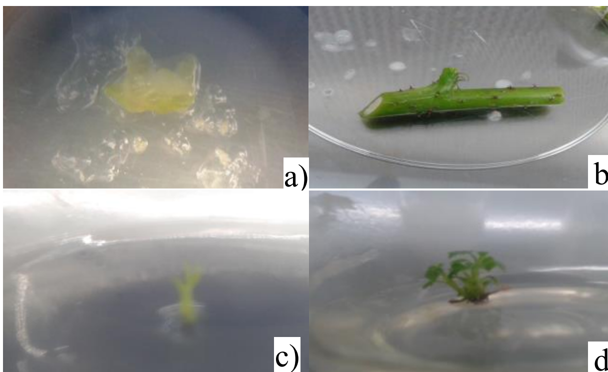

Figure 5. Raspberry vegetative material *cv* Heritage; a) meristematic tissue observed in 2x stereoscope microscope; b) raspberry internode, disinfected and ready to be planted; c) explant from meristematic tissue in full growth; d) explant in growth from nodal segment.

Conclusions

In strawberry *cv* Aromas, *in vitro* establishment, based on apices, was successful in the four culture media evaluated, since it was possible to establish them, although TT1 proved to have the greatest viability, as it had the highest percentage of vegetative tissue. stable with 70% and the lowest percentage with TT4, with only 20%. The combination of hormones in the formulation allowed the successful development of the tissue for its *in vitro* establishment of strawberry (Folta, *et al*., 2006).

In all treatments, the percentage of contamination did not exceed 30%, considering, as well as acceptable for an *in vitro* propagation system.

For the case of raspberry, *cv* Heritage, the formulation proposed by Anderson (1980) and Sigarroa and García (2011) was the only one that allowed the development of the explants, there being a greater percentage of establishment by the technique where internodes schemes were used, but not by medium of meristems, where there was a high rate of necrosation and death of the established tissue, in the other treatments. The formulation with the combination of BAP + GA hormones, without the presence of auxin AIB, was more successful in the establishment of nodal segments in raspberry.

On the other hand, when meristem cells were extracted, it showed a much lower percentage of contamination compared to the explants obtained by sowing internodes, because the disinfection system is more successful when it comes to extracting tissue of the cambium (Yildiz, 2012). When the meristems were evaluated, a high percentage of tissue necrosis was obtained, but the percentage of contamination was very low. Therefore, it is suggested to evaluate this technique again, using another type of disinfectant agents and tissue exposure times before them.

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