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Obtaining Protoplasts from Leaf Tissue Plantlets of *Rubus glaucus* Benth (Blackberry) to Develop Proembryos

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Abstract

Blackberry is a native plant from the highlands of South America, which grows in the Ecuadorian Andes. This plant has been cultivated for centuries in Ecuador and used as food and medicine. Its fruit is widely consumed due to its high content of minerals and vitamins, especially vitamin C. For a large-scale production, this study was aimed to establish alternatives and appropriate protocols for obtaining proembryos of *Rubus glaucus* using callus and protoplasts from leaf tissues. The following hormones were used in different concentrations: Auxins (2,4-D and NAA), Cytokinins (TDZ and Zeatine) and Giberillin GA3 and a basic Murashige and Skoog (MS) media as culture medium. Several assays were performed to determine the most suitable concentration of phytohormons for embryogenic callus formation and for stablishing proembryos in cell suspensions. Statistical analysis did with the help of Analysis of Variance (ANOVA) and Duncan test. The best treatment ($p < 0.0001$) was reached with 0.5 ppm of 2, 4-D auxin in Callogenesis phase, this concentration was sufficient to form vegetative callus over a period of four weeks with the leaf explant covered totally. Regarding to the increase of vegetative growth of callus, the cytokinin Zeatin at 0.5 ppm enabled an average increase of 0.5 cm per growing callus and finally 0.5 ppm of GA3 enabled an average growth rate of 141, 667 cells/mL in the protoplast suspension.

Keywords: Blackberry, Leaf Tissue, Plantlets, Proembryos, Protoplasts

1. Introduction

Rubus glaucus Benth, known as Blackberry, is an Andean plant that is distributed throughout Andean region of Ecuador and elsewhere in Subtropical America, has great commercial importance; it has been cultivated in the country for more than seven decades in regions where the altitude ranges from 1200-3000 meters[1]. Because of its major commercial importance and wide cultivation in Ecuador, it is widely grown in Inter-Andean valleys in the foothills of the Sierra, in lands located between 2,500 and 3,000 meters, at temperatures between 12-18°C and rainfall of 600 to 800mm. This fruit is produced mainly in the provinces of Tungurahua, Cotopaxi, Carchi, Imbabura, Pichincha and Bolivar. Domestic blackberry production is constantly increasing[1,2]. The varieties commonly used in these areas correspond to Blackberry and Blackberry Brazos. But this production only meets the demand of domestic market but not the external market. Blackberry is grown mainly in an area of more than 5200 hectares with a production of 12 to 14 tons per year, this production supply only domestic market, but not enough to supply the external market[3]. Ecuador in 2008 exported 11.93 tons of blackberry to worldwide market,

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the main destinations were EEUU, Spain, Netherlands Antilles and Germany[4,5]. Currently in Ecuador there has been an increase of 3% in the consumption of blackberry for their ability to canning and acceptance to consume fresh. The blackberry is a fruit of cold weather has improved its projection in recent years due to increased demand in the domestic market, the potential for export and continuity of income that cultivation represents for the small producer. According to Monteiro[7] the plant is propagated by traditional vegetative methods, cuttings and layering which are economic methods, but have the disadvantage that they are not very suitable and often cause spread of pests and diseases affecting plant quality. In Ecuador there are no better alternatives developed to ensure adequate production volumes and excellent quality plants. Propagation in vitro is part of the modernization of agriculture, the application of these techniques contribute to improving health conditions of the plantation and increase their productivity. The optimization of protocols for cell culture starting from somatic cells holds great promise for the use of this technology in breeding processes in blackberry; and for the rescue of genes of interest for growers and for the production of certified plants adapt better to the environment and allowing them to efficiently combat various pests and diseases, reducing the use of pesticides that affect the planet.

A suitable alternative method that ensures the development of healthy plants and superior genetic potential is in vitro culture. It is described as a process by which any vegetative part (explant), under aseptic conditions, in the presence of nutrients and plant hormones, plant regenerates a new clone, free of viruses and diseases and especially high quality. In addition clonal plants are propagated in any season, in small spaces and can multiply in large quantities from some vegetative material[8]. Various techniques have been developed for growing plantlets, including: micro graft, micro piles culture, apical explants of leaf segments, somatic embryos, pre-embryos, pollen grains, embryos and protoplasts. This technique is known as somatic embryogenesis and according as Freire[9], in vitro somatic embryogenesis it is possible since virtually any plant somatic tissue has the capacity to develop into an embryo (Totipotency) through manipulation of culture conditions and the application of growth regulators. Perez[10] indicates that the best alternative method to produce plantlets on a large scale is through indirect means, for which an intermediate callus phase consists of a set of different types of unorganized cells is required and conserving the ability to divide. Once callus vegetative organized this proliferates the formation of proembryos, usually begins in a culture medium containing high concentrations of auxin and then the calli were transferred to a culture medium with lower concentrations of plant growth regulators to induce the formation of somatic embryos from initial proembryos. Vegetative calluses are defined as amorphous masses that develop as a result of cell proliferation parenchymatous tissue. It is often the consequence of a wound; it can be located on a stem, root and leaf. Calluses have no predictable patterns of organization and an important feature is its irregular growth, with the potential to develop normal roots, shoots and seedlings forming embryos[11] Calluses under controlled conditions of nutrients and growth regulators and simultaneously develop proembyos are a source of obtaining protoplasts grown in liquid media can generate huge amounts of stem cells capable of differentiating into new plants. The cultivation of cells in suspension is a set of isolated cells and cell aggregates (2.0-100 cells) are distributed in a liquid culture medium in constant motion[9]. Protoplasts within the same culture have a wide variety of shapes and sizes, from spherical to cylindrical. The dimensions of these cells range from 20 to 40um in diameter and 30 to 200um length[12]. In Ecuador there has been little research on blackberry micro propagation. No studies on plant regeneration are recorded from cell suspension cultures (protoplasts) using leaf tissue or somatic embryogenesis of undifferentiated tissue (callus plant). Currently many researchers[13] reported that the development of embryogenic cell suspensions opens up the possibility for mass production of plants at low cost and with good results. As far as so many researchers develop different methods to obtain pro embryos in Blackberry. But in Ecuador there is no alternative method develops for mass production of plants by cultivation of protoplasts (cells without cell wall) and subsequent pro embryos development. So in this investigation we try to establish alternative and appropriate protocols for obtaining vegetative pro embryos from leaf tissue of blackberry plantlets, *Rubus glaucus* Benth.

2. Materials and Methods

2.1 Location

The research was undertake in Biotechnology Laboratory, located in El Prado, Agricultural Sciences Faculty (IASA), Universidad de las Fuerzas (ESPE), Ecuador.

2.2 Step of Obtaining Plant Material

Plant material from *Rubus glaucus* Benth (Blackberry) mother plants were obtained from greenhouse. For multiplication, stem segments with a knot was used, they were placed on Murashige and Skoog, (MS) basal medium supplemented with 100 mgxl^{-1} Myoinositol, 150 mg x L^{-1} PVP, 50 mg x l^{-1} ascorbic acid, 2 ppm BAP, 0.5 ppm AIA, 3% sucrose and 0.7% agar. The medium pH was set to 5.8 before autoclaving. Once plantlets regenerated, leaf explants were planted on MS media to regenerate complete plantlets. The obtained plantlets were kept in tissue culture laboratory. Every three weeks, Blackberry plants were cultivated in vitro to obtain an adequate amount of plants needed to provide the number of sheets needed to regenerate them calluses or pro embryos and multiply in liquid culture media to subsequently perform multiplication and plant regeneration mass.

2.3 Plant Material and Embryogenic Callus Induction

2.3.1 First phase

Vegetative explants were collected from Blackberry plantlets; healthy explants were sterilized and planted on Murashige-Skoog media (MS). At the end of the fourth week of in vitro vegetative growth and according Dohm et al.[14], Blackberry leaves were sectioned at the base, they underwent several parallel cuts with 1mm apart and placed in Petri dishes with induction nutrient medium.

At this stage, several assays were performed to determine the most suitable concentration of Phytohormones for embryogenic callus formation, a basic Murashige and Skoog (MS) 100% was used, to which was added different concentrations of auxin (2, 4D and ANA).

2.3.2 Second Stage

After four weeks, callus were moved to medium (MS) 100% supplemented with different concentrations of Thidiazuron (TDZ) and zeatin to cause increase in size of the embryogenic callus obtained in the preceding stage. All assays were exposed for three weeks to light and controlled temperature of 25°C.

2.4 Culturing Cells in Suspension

At this stage, several assays were performed to determine best phytohormone cocentración to cell suspension. It began with the incubation of pieces of friable callus obtained from the previous phase in liquid MS media + ANA + zéatine + different conectrations of GA3 (0, 0.1, 0.5, and 1 ppm) Aguilar et al.[16] and Roca[17]. All friable callus obtained from the previous phase were performed fine cuts to help the cell detachment and get protoplasts in less time. Protoplasts were included in culture cell flasks with liquid medium and placed in shaker-incubator at 25°C and 90 rpm. Cell suspension was filtered in fine pore membrane in order to remove those cell components which do not meet the optimum characteristics to create a cell suspension[16]. To set the cell growth dynamics direct counting in a Neubauer chamber needed to be done using an optical microscope for each interval evaluation. Daily record for nine days was performed.

3. Statistical Analysis

The Design was Completely Random (DCA) with a factorial arrangement A x B where A has two levels and factor B has 4 levels. The experimental unit was a group of five Petri dishes on which seven treatments were applied, according to the levels of concentration and type of auxin applied. Data were analyzed by ANOVA with a Duncan test at 5% to verify the best auxin and the best concentration to favor the induction of embryogenic callus formation[24].

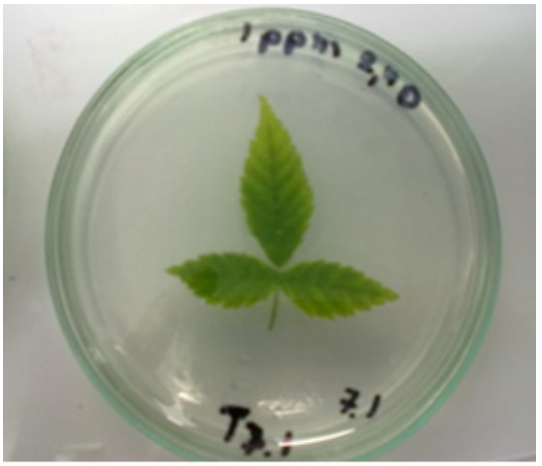
4. Results and Discussion

4.1 Callus Induction (Callogenesis)

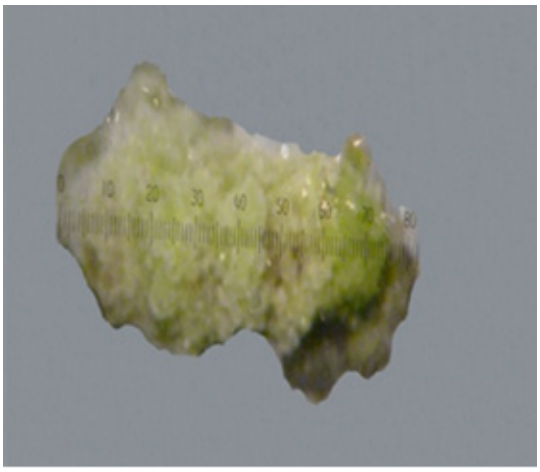
4.1.1 First Phase

The initial callus induction was achieved only from fresh leaf explants from plantlets of Blackberry. For induction of friable callus, parallel cuts on the leaf of blackberry were very important, better contact of hormones and further development of calluses in shorter periods of time was achieved, which is corroborated by the methodology described by Dohm et al.[14]. For better cell proliferation different concentrations of Phytohormones BAP + 2.4D and BAP + ANA were tested, this interaction the best cell proliferation was achieved BAP + 2.4D, mentioned coincides with the work done by Hurtado & Merino[12]. Dohm et al.[14] obtained callus at 8 and 6 weeks using 0.022 ppm and 1 ppm concentrations of 2,4-D hormone

respectively. These results do not agree with those obtained in the present investigation, since the second week an average of seven callus in T2 treatment was obtained (MS + 2, 4-D 0.5 ppm), opposed to the other treatments in which an average of three callus was obtained and at the end of the fourth week in the same treatment calluses occupied 100% leaf explant, see Figures 1 and 2.



(a)



(b)

Figure 1. Induction Callus (**a**) First week, (**b**) Fourth week.

In establishing the analysis of variance for the number of calluses in the nutrient medium with auxin induction, a statistical difference in treatment 2 was found to a level of 5%. Duncan test determined that for auxin induction callus, the best treatment was T2 containing 0.5ppm of 2,4-D, followed by T3 and T7 treatments at concentrations of 1 ppm and 1 ppm of 2,4-D ANA respectively. T2 treatment produced more callus (48) until the fourth week, unlike T7 and T3 which produced 24 and 20 each callus. See Table 1.

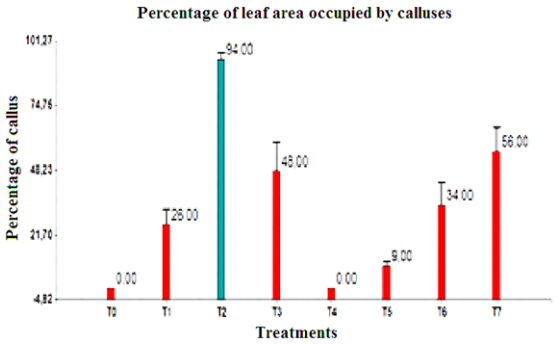


Figure 2. Development of callus induction phase.

Table 1. Test: Duncan Alfa = 0,05

Error: 224, 3750 fd: 32				
Tratments	**Average**	**n**	**E.E.**	
T4	0,00	5	6,70	A
T0	0,00	5	6,70	A
T5	9,00	5	6,70	A B
T1	26,00	5	6,70	B C
T6	34,00	5	6,70	C D
T3	48,00	5	6,7	D E
T7	56,00	5	6,70	E
T2	94,00	5	6,70	F

Averages with common letter are not significantly different (p < = 0.05)

MS + ANA medium used in the research suggests that the best concentration of ANA is 1ppm and allowed to obtain calluses at 2 weeks. These positive results were obtained by subjecting treatments darkness that contrasts with the results obtained by Rivera and Perea[18], which used 16-hour light photoperiod obtaining callus formation at 4 weeks. From the above it is shown that auxin are sensitive to light and as photoperiod increases plant hormones are inactivated.

All treatments were allowed four weeks in darkness to prevent degrade auxins and temperature controlled at 25°C incubation to prevent phenolization (release of oxidizing compounds) and death of the explants[15, 12]. See Figure 3.

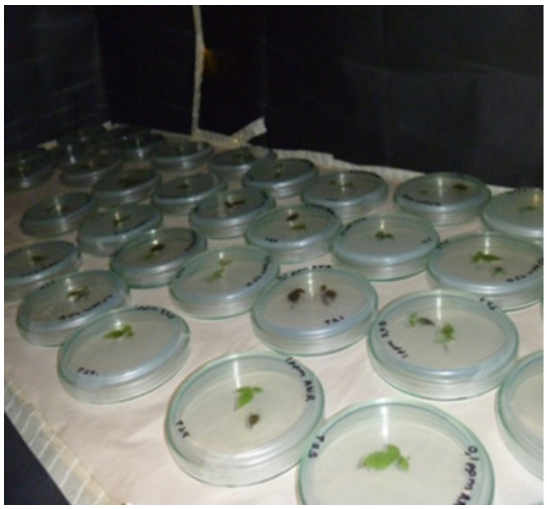
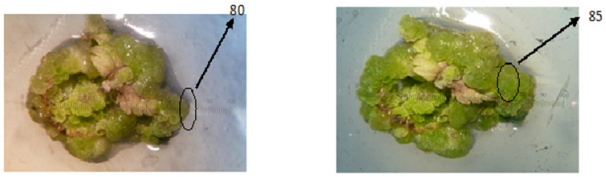


Figure 3. Culture Media darkroom induction.

4.1.2 Second stage

The callus growth began in the second week in treating T6 containing MS + 0.5 ppm of with 24 hours photoperiod, at the end of the third week an average increase of 0.5 cm in callus size was recorded, see Figure 4. Unlike obtained in research by Zhou et al.[19] which states that the best dose was 0.23 ppm zeatine and 18 hours light photoperiod and 6 hours of darkness, achieving growth of 2 cm in 8 weeks. Similar research in banana and plantain, Aguilar et al.[16] mentioned that to achieve further development and growth of callus is necessary to use Cytokinins in the presence of light. In this research it was achieved using concentrations of 2, 4-D 0.5 ppm + Zeatine 0.5ppm and 24 hours of photoperiods.



(a) **(b)**

Figure 4. Callus growth in T6 (**a**) In the second week, (**b**) In the third week. 200X magnification.

By setting the variance analysis for the size of calluses obtained in growth medium containing Cytokinins, statistical differences between treatments was found. An F 33.25 and $p < 0.0001$ at a level of 5% error was obtained. Regarding the increase in size of vegetative callus using Cytokinins, the best treatment was achieved with T6 treatment containing 0.5ppm Zeatin, this treatment increased from 0.4 to 0.5 cm per week were recorded. T2 and T3 to 0.5ppm and 1 ppm concentrations of TDZ respectively, increased from 0.1 to 0.3cm per week. The increase in size was assessed by Duncan statistical test. See Figure 5.

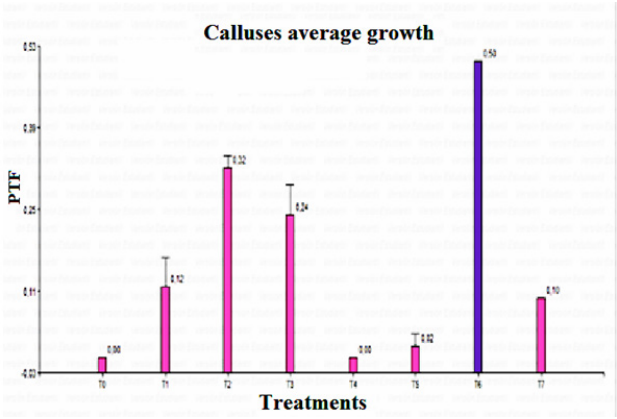


Figure 5. Treatments evaluated for increasing the size of calluses in Phase 1.

The result shown in colorimetry confirms that friable calli appeared in the T6 treatment (MS + 0.5ppm Zeatin), which were used to proceed to the next phase. See Figures 6 and 7.

Figure 6. Friable calluses T6 (MS + 0.5 ppm Zeatin). 200X Magnificatio.

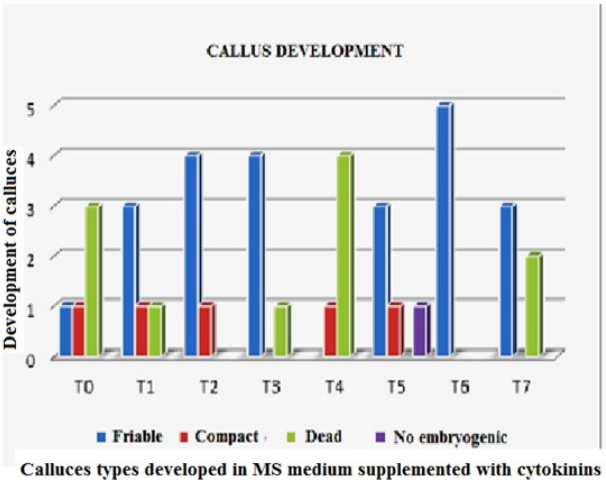


Figure 7. Types of calluses obtained in callus formation phase.

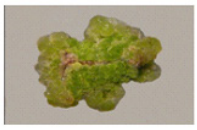
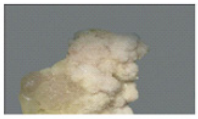
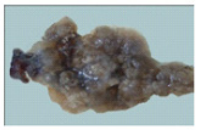
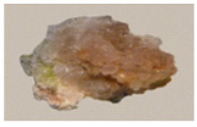
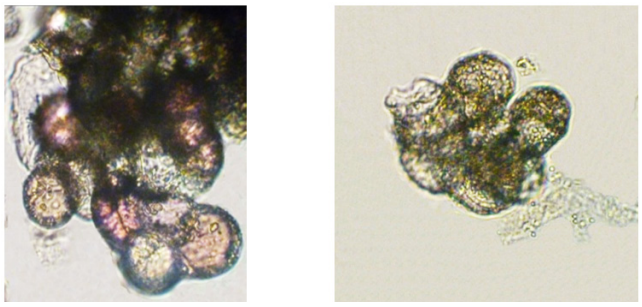
Callus	Coloration	Photography
Friable	Green	
Compact type 1	Crystal color	
Compact type 2	Whitish	
Dead	Blacking	
No embryogenic	Brown	

Figure 8. Callus types

The above figure details the type of callus obtained at each treatment after exposure to the nutrient medium enriched with cytokinins. All treatments developed friable calluses with 57.5%, followed by the dead calluses with 27.5%, 12.5% compact and finally no embryogenic callus which reached 2.5%. The best treatment was T6 with 100%, followed by treatment T2 and T3 with 80% of friable calluses. Regarding control treatments where cytokinins not used, not development of calluses was obtained as proven in work by Chuyn et al.[20], using in *Matricaria recutita* L, where no callus growth was achieved. Everything mentioned above demonstrates that cytokinins are plant hormones that stimulate cell growth or division. See Figure 8.

4.2 Protoplast Culture

To accumulate adequate amounts of protoplasts was necessary to make several parallel cuts 1 mm apart in the leaf tissue, subjecting vacuum, constantly stirring and incubating with enzymes, indicated agrees with the work done by De la Torre[21] and Osorio et al.[22]. In this study the number of protoplasts per mL was 141,667 cells/ml. This value was minimal compared to the work done by De la Torre[21], who obtained a concentration of $2.41x10^5$ protoplasts obtained from rose. This difference in the amount of protoplasts in the two plant species although located within the same family is mainly due to: the blackberry leafs has different characteristics, greater amount of cuticle, more lignified leaves, higher percentage of lignin and cellulose which are essential factors influencing directly to obtain adequate amounts of protoplasts. See Figure 9.



(a) **(b)**

Figure 9. (**a**)Initial detachment protoplasts, (**b**) Spherical protoplasts.

Protoplasts obtained were placed in a nutrient medium MS+GA3 5 ppm. with this concentration 45.833 protoplasts was obtained at first day, at 3 and 4 days protoplasts formed groups by 20% and on the 5th day cell growth stopped, which demonstrate that the gibberellic acid stimulates cell growth. Similar data were obtained in

studies conducted by Andreu et al.[23], for culturing plum plant protoplasts and it was performed in a medium with half macronutrient concentrations without pyridoxine and nicotinic acid was added Hydrolyzate casein, sucrose, mannitol, ANA and BAP 3-4 days getting to the initiation of cell division, 6 days a percent found between 43 and 70% of dividing protoplasts and 14 days most protoplasts had formed groups of 3 cells but thereafter, divisions stopped. All protoplasts obtained were placed in MS + GA3 5 ppm nutrient medium. T2 treatment, achieved the highest multiplication with a total average of 141,667 cells / ml on the fourth day. Similar data were obtained in studies conducted by Andreu et al.[23]. Figure 10.

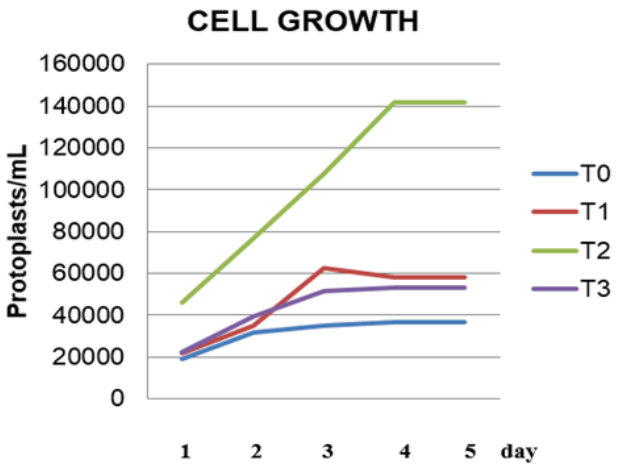


Figure 10. Cell growth in MS + GA3 medium.

As shown in the previous figure with T2 treatment greatest cell multiplication was obtained, unlike the treatment T1 from three day decreased cell concentration and treatment T0 cell multiplication is minimal. See Figure 11.

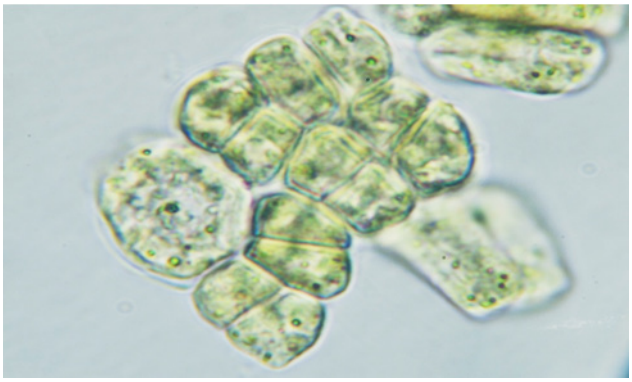


Figure 11. Cell multiplication (protoplasts) 400X magnification.

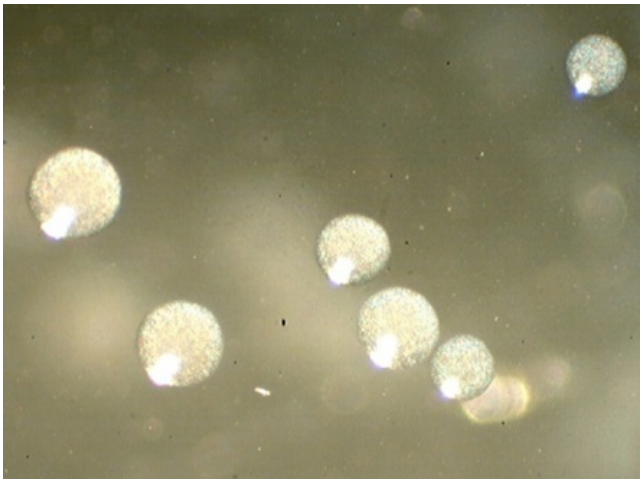


Figure 12. Proembryos derived from protoplasts. 100 X magnification.

To confirm the viability and regeneration capacity to initial callus, protoplasts obtained were seeded in solid medium (MS + 0.5ppm 2.4D). In two weeks microcalli formation was achieved, at week 3 white crystal callus, in the fourth week it was possible to obtain compact callus and growth continued to form compact proembryos were developed. Figure 12 Comparing these results with those obtained by Andreu et al.[23] despite using 2.4D Phytohormone that stimulates callus formation, it failed to obtain proembryos. See Figure 12.

5. Conclusions

Young leaves of blackberry from in vitro plants are the best plant material to generate callus formation. 2,4D Phytohormone in 0.5 ppm concentration was the best treatment to induce a significant number of friable calluses. Zeatin concentration of 0.5 ppm and 24 hours photo period allowed development of viable callus up to 5 cm. Gibberellic acid in concentration of 0.5 ppm allow adequate cellular adaptation in the liquid culture medium, easy release protoplasts and subsequent development proembryos microcalluses somatic differentiation in nutritious gels containing 0.5 ppm MS + 2.4D.

The protocol for obtaining proembryos and microcalluses developing from leaf tissue was MS medium + 2.4 D 0.5ppm, followed of zeatine 0.5ppm and finally for cell establishment MS + 0.5 ppm GA3.

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