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Proceeding Paper

Changes of Secondary Metabolites during Tamarillo Somatic Embryogenesis †

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Abstract: Tamarillo (Solanum betaceum Cav.) is a Solanaceae tree cultivated for its edible fruits. Under specific stimuli, indirect somatic embryogenesis (SE) originates distinct calli: embryogenic (EC) and non-embryogenic (NEC). Both types proliferate, but only EC originates somatic embryos. The presence of secondary metabolites is known to influence induction and embryogenic competence. The objective of this work is to study some of these compounds on SE. Results showed highest dedifferentiation rates (>90%) in the control and when rutin and caffeic acid were tested, whereas lower concentrations inhibit dedifferentiation. In contrast, the compounds seem to inhibit growth of established calli without affecting protein, phenolic acids and flavonoid levels, measured by spectrophotometric methods. Anthranilic acid completely inhibited both induction and calli growth. The results seem to present a correlation of some secondary metabolites with dedifferentiation rates during induction and a tendency to inhibit growth of established calli, probably related to metabolic effects. Further studies are underway to further characterize the dose-response relation of these compounds and molecular mechanism underlying this phenotypic effect.

Keywords: *Calli*; embryogenic competence; flavonoids; phenolic acids

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1. Introduction

Tamarillo (*Solanum betaceum* Cav.) is a Solanaceae tree, indigenous to South America, cultivated globally due to its edible fruits [1]. The economic importance of tamarillo, along with the problems associated with traditional propagation methods, has led to the development of several in vitro biotechnological cloning methodologies, particularly somatic embryogenesis (SE) [2,3].

In general, SE is a process by which a somatic tissue or cell develops into a structure similar to a zygotic embryo without fecundation [4]. In tamarillo, SE can be achieved by two different pathways that emerge from distinct initial explants and the cultures conditions applied as reviewed in [3]. One methodology that has been extensively studied and optimized is the dedifferentiation of leaf segments in an auxin rich media with the formation of two types of callus tissue, embryogenic and non-embryogenic (EC and NEC, respectively) that can be distinguished by morphologic characteristics, and embryogenic competence. Upon removal of auxin from the culture medium. In these conditions, EC develops into somatic embryos that can be germinated while NEC becomes necrotic. Therefore, this protocol can be divided into two steps, the induction of embryogenic tissue cells from the initial explant and the development of the appropriate callus into somatic embryos [2].

SE is a complex process influenced by several endogenous and exogenous factors. One factor that has been related to the induction phase, particularly the early dedifferentiation phase is the presence of certain types of secondary metabolites, such as phenolic acids and flavonoids, which can improve induction and to some degree hinder somatic embryo formation [5]. The objective of the present work is to analyse the influence of some of these compounds in tamarillo SE. (optional).

2. Materials and Methods

2.1. Plant Material and Culture Conditions

The leaves used for the induction experiments were obtained from tamarillo clones previously established in vitro and maintained in a Murashige-Skoog medium [6] supplemented with 8.6 mM of sucrose and 0.88 μM of 6-Benzylaminopurine. The pH was adjusted to 5.7 before addition of agar (6 g/L). Shoot cultures were maintained in a culture chamber 25 °C, in a 16 h photoperiod, at 25–35 μmol m-2s-1 (white cool fluorescent lamps).

2.2. Somatic Embryogeneis Induction and Secondary Metabolite Quantification

For the induction of embryogenic tisse, leaf segments (about 5 mm in diameter, around 25 per Petri dish) were aseptically removed from the micropropagated shoot and placed with the abaxial part in direct contact with the induction medium (Murashige-Skoog medium supplemented with 26 mM of sucrose and 20 μM of picloram – TP medium). The pH was again adjusted to 5.7 before adding the gelling agent (2.5 g/L of Phytagel™, Sigma). To study the influence of phenolic acids caffeic acid (896 and 448 μM) was used, while flavonoids were assayed with rutin (197.5 and 98.75μM). Anthranilic acid, an amidobenzoic acid, was also tested (1164.8 and 582.4 μM). The secondary metabolites were prepared by dissolution in a minimum amount of ethanol followed by distilled water, sterilized by filtration (0.45 μm cellulose filter) and added to the medium after autoclaving (121 °C; 20 min).

After 8 weeks of culture, the dedifferentiation percentage explants producing calli was recorded.

Additionally, previously established EC and NEC lines, induced and proliferated in TP medium, were tested in liquid medium under culture conditions previously reported [7] with either 896 μM of caffeic acid, 197.5 μM of rutin or 1164.8 μM of anthranilic acid. After 3 weeks, growth rate (final mass – initial mass/3) intracellular concentration of phenolic acids and of flavonoids were determined. Total phenolic acids and flavonoids were assayed by the methodology described in [8]. Briefly, plant material was extracted with methanol (1 g.f.w: 1 mL), centrifuged (10 000 g: 10 min) and the supernatant analysed. For phenolic acids 10 μL of samples were used and the reaction was initiated with the addition of 100 μL 1:10 Folin-Ciocalteu reagent (Sigma) followed by 80μL of 1M Na2NO2). A calibration curve was prepared with caffeic acid in concentrations between 25 and 150 μg/mL, the absorbance read at 630 nm, and the results presented as μg of equivalents of caffeic acid per ml (μg eCA/mL). Flavonoids were estimated using 25 μL of sample to which 100 μL distilled water, 10μL of NaNO2 (50 g/L), 15 μL of AlCl3 (400 g/L) and 50μL of 1M NaOH were added. A rutin calibration curve was prepared with concentrations between 25 and 150 μg/mL, absorbance was read at 510 nm and the results presented as μg of equivalents of rutin per millilitre (μg eR/mL).

2.3. Statistical Analysis

Homogeneity of variances was tested using the Brown-Forsythe test. In the case of homogeneity of variances, ANOVA was carried out and, where necessary, means were compared using Tukey post hoc test ($p < 0.05$). If homogeneity of variances was not confirmed, the non-parametric Kruskal-Wallis test was used and in this case Dunn's multiple comparisons was the post hoc test ($p < 0.05$).

3. Results and Discussion

3.1. Induction Phase

The callus induction of the initial explants was measured after 8 weeks of growth and represented as percentage of dedifferentiation (Figure 1).

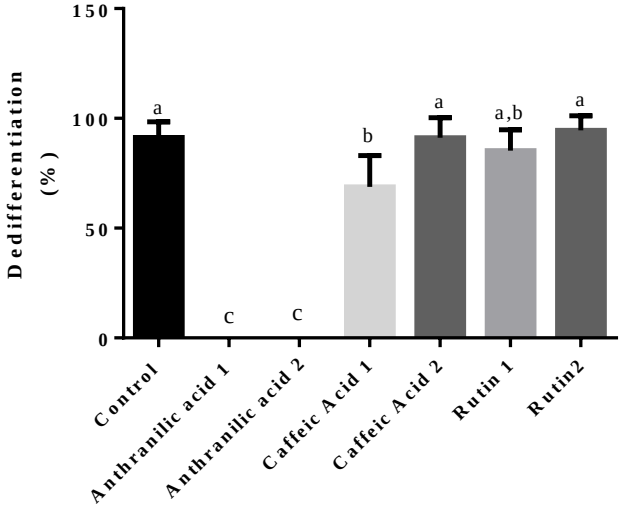

Figure 1. Dedifferentiation percentages of the initial explants. Results are presented as mean ± standard deviation (SD; $n = 5$). 1 is the highest concentration of compound used. Different letters indicate statistically significant results by Tukey's test ($p < 0.05$).

The highest induction occurred in the control group, and in the lower concentrations of caffeic acid and rutin showed the lowest induction capacity. The highest concentration of rutin presents an intermediate result while the highest caffeic acid concentration presents a significantly lower dedifferentiation. Anthranilic acid, regardless of the concentration applied, totally inhibited any callus formation. Other authors have reported that callus induction is a phenomena closely related to phenolic compound production [9] an these compounds can influence the induction and/or maturation of somatic embryos [5]. The results presented here support these observations, and the response appears to be that of a classic dose-response curve, which should be further investigated. The molecular basis of this response is also at the present unclear but may be closely related to stress responses, as phenolic and flavonoid compounds are related to stressors factors, particularly abiotic, and SE is a phenomena related with stress [4,10,11].

Anthranilic acid completely inhibited callus induction. This compound is a precursor of several important biosynthetic pathways, specifically those dependent of tryptophan such as the synthesis of the auxin indol-3-acetic acid (IAA) and some alkaloids [12,13]. As the culture medium is enriched with picloram, a strong auxin, the inhibition may be due to accumulation of toxic levels of auxin or auxin-like compounds. Currently, studies are being carried out to study this hypothesis.

3.2. EC and NEC Growth

Previously established EC and NEC lines were grown in the conditions previously reported [7]. Liquid TP medium was used as control, and one concentration of each compound as tested: anthranilic acid (1164.8 μM), caffeic acid (896 μM) and rutin (197.5 μM). After 3 weeks, final masses were registered and growth rates calculated (Figure 2).

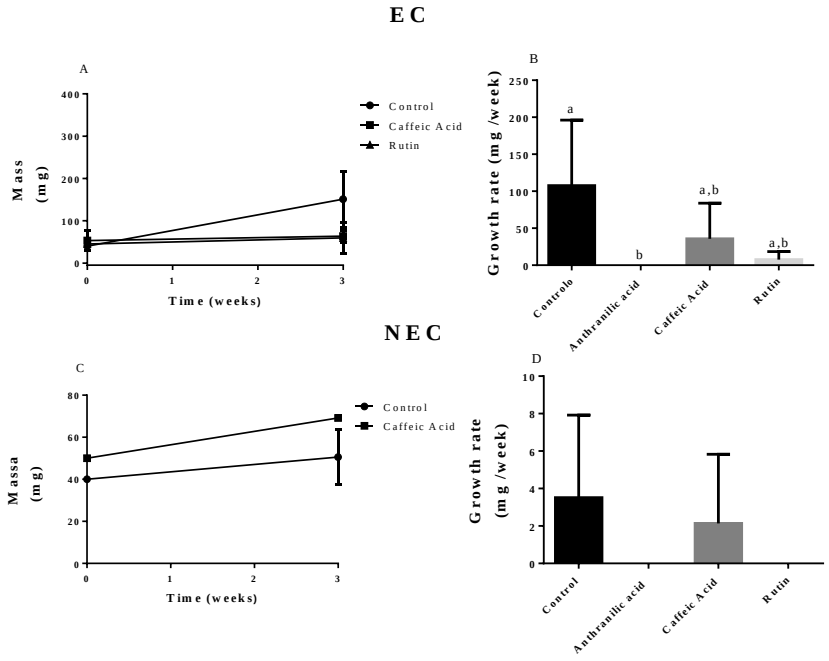

Figure 2. Callus growth in liquid medium. (A) Mass increment of EC; (B) Growth rates of EC; (C) Mass increment of NEC; (D) Growth rates of NEC. Results are presented as mean ± SD. Different letters indicate statistically significant results by Dunn's multiple comparison test ($p < 0.05$).

In terms of EC, the control group showed the largest growth rates, albeit statistically insignificant from caffeic acid and rutin. Anthranilic acid completely inhibited the growth (final mass of filtrate was equal or lower than initial mass). In terms of NEC, only control and caffeic acid showed any measurable mass increment, with growth rates for the other compounds considered null.

Finally, the intracellular content of phenolic acids and flavonoids was assayed in each cell line (Figure 3).

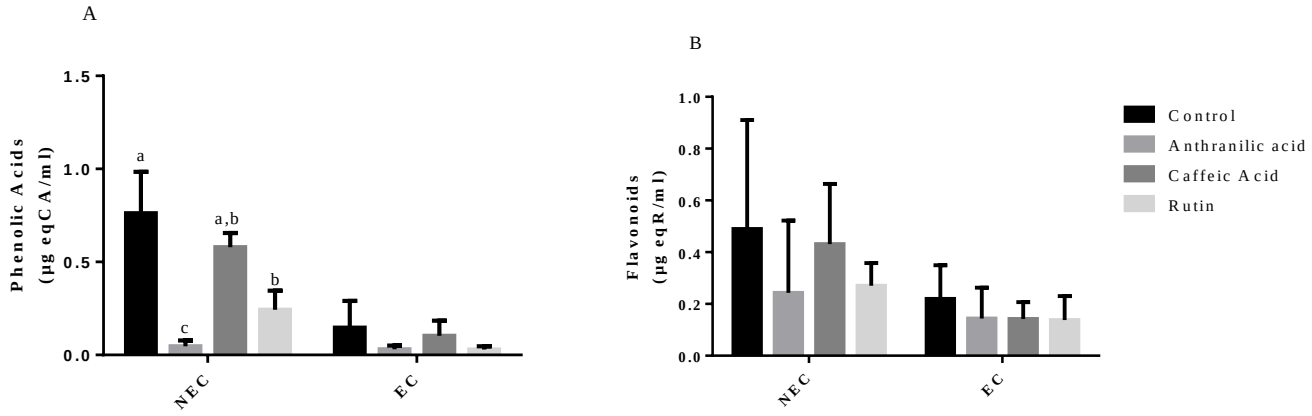

Figure 3. Total content of phenolic acids (A) and flavonoids (B) in both types of callus assayed. Results are presented as mean ± SD. No statistically significant different results were found by Tukey's test ($p < 0.05$).

The phenolic content of NEC is the highest of phenolic acids, with caffeic acid presenting an intermediate value, rutin and anthranilic the lowest values (Figure 3). These results could be explained in part by the lower growth of the rutin and anthranilic acid

samples. On the other hand the lower concentration of total intracellular phenolic acids in the presence of caffeic acid seems to suggest that this factor is somewhat independent of extracellular concentrations. In EC no differences in phenolic acids were observed, while flavonoid concentration seems to be unrelated to the growth conditions, although the quantification method might be improper). Overall, the results seem to indicate that the presence of phenolic acids and flavonoids in the extracellular medium is detrimental for tamarillo callus growth and there seems to be little intracellular transport as cells grown in phenolic or flavonoid rich media di not present a higher intracellular concentration. although some studies have shown that in vitro plants are a potential good system for the production of certain flavonoids [14], the influence of these family of compounds needs to be further investigated in the system presented here.

4. Conclusion

The present work describes the influence of certain types of secondary metabolites, namely phenolic acids and flavonoids, during tamarillo SE. The results show that these compounds have some importance in the induction phase while seem to be disadvantageous in the multiplication of EC and NEC masses in liquid medium. Further studies are underway to understand their impact on other stages of the in vitro system. Also, future studies should also focus on a more precise quantification of these compounds in the system and the molecular mechanism of action.

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