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TAMARILLO (*SOLANUM BETACEUM*) CELL LINES CULTURE OPTIMIZATION FOR PLANT-DERIVED METABOLITES PRODUCTION

Dissertação no âmbito do Mestrado em Bioquímica orientada pelas Professoras Doutoras Paula Cristina Veríssimo Pires e Sandra Isabel Marques Correia pertencentes à Faculdade de Ciências e Tecnologia.

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Abstract

Solanum betaceum Cav., commonly named tamarillo, belongs to the solanaceous family and is a southern America native plant. Tamarillo can be micropropagated through somatic embryogenesis (SE), a process in which embryogenic and non-embryogenic cell lines can be induced from different explants. Two non-embryogenic cellular lines were studied in this work: non-embryogenic cellular line 1 (NEL 1) and non-embryogenic cellular line 2 (NEL 2). These type of cell lines can be applied to biotechnological systems, named plant cell suspension cultures, in order to study and analyze cellular and molecular processes in a variety of plant species, and are currently used to produce important products like recombinant proteins and secondary metabolites. Furthermore, these products can be produce in large-scale in liquid sterile environments named bioreactors.

Some biotechnological approaches have been made for the enhancing of products production by plants and, the use of elicitors is one of them. The use of elicitors in plant cell suspension cultures may cause several biological effects like an increase in the production of secondary metabolites.

The objective of this work was to characterize and optimize tamarillo cellular lines cultures using a bioreactor system in terms of: cellular growth, metabolites production and two elicitors (yeast extract and casein hydrolysed) influence. For that cellular growth curves, protein extracts and profiles and proteolytic activities were obtained and analysed.

The results obtained determined optimal growth conditions for both cell lines analysed, and showed that the sucrose concentration in the liquid medium affected the production of different proteins in both cellular lines. Moreover, the addition of yeast extract elicitor influenced the production of glycosidase hydrolases and alkaline phosphates, in both cellular lines. Casein hydrolyzed influenced the proteases enhance, more in NEL 2 than in NEL 1.

Key-words: somatic embryogenesis; plant cell suspension cultures; products production; bioreactors; elicitors.

Resumo

Solanum betaceum Cav., normalmente designado por tamarilho, é uma espécie da família das solanáceas e é uma planta nativa do sul da América. O tamarilho por ser micropropagado através da técnica de embriogénese somática (SE), que permite a indução de linhas celulares embriogénicas e não embriogénicas. Duas linhas celulares não-embriogénicas foram estudadas neste trabalho: linha celular não-embriogénica 1 (NEL 1) e linha celular não-embriogénica 2 (NEL 2). Este tipo de linhas celulares pode ser aplicado a sistemas biotecnológicos, como a cultura de células vegetais em suspensão, com o objetivo de estudar e analisar processos moleculares e celulares numa variedade de espécies de plantas. Além disso, estes sistemas são utilizados para produzir proteínas recombinantes e metabolitos secundários que podem ser produzidos em larga escala em bioreatores.

Algumas metodologias biotecnológicas têm sido aplicadas para aumentar a produção dos produtos oriundos de sistemas vegetais, sendo o uso de elicitadores uma dessas estratégias. O uso de elicitadores nas culturas de células vegetais em suspensão pode causar diversos efeitos biológicos como o aumento da produção de metabolitos secundários.

O objetivo principal deste trabalho foi caracterizar e otimizar linhas celulares de tamarilho em cultura, usando um sistema de bioreator, em termos de: crescimento celular, produção de metabolitos e influência de dois elicitadores (extrato de levedura e hidrolisado de caseína) nas culturas. Para tal obteve-se curvas de crescimento celular, extratos e perfis proteicos e perfis proteolíticos para as diferentes condições em estudo.

Os resultados obtidos permitiram determinar condições de crescimento ótimas para as duas linhas celulares em estudo e mostraram que a concentração de sucrose presente no meio líquido afetou a produção de diferentes proteínas, nas duas linhas celulares. A adição de extrato de levedura influenciou a produção de hidrolases glicosídicas e fosfatases alcalinas. O hidrolisado de caseína influenciou o aumento de protéases, mais para a NEL 2 do que para a NEL 1.

Palavras-chave: embriogénese somática; culturas de células vegetais em suspensão; produção de produtos; bioreatores; elicitadores.

1. Introduction

1.1 Tamarillo

Solanum betaceum Cav., commonly called tamarillo, belongs to the solanaceous family and is a southern America native plant. The exact origin location of tamarillo is not known but some reports indicate the appearance of this tree in southern Bolivia and northeastern Argentina (Prohens and Nuez 2010). The original name for tamarillo was tree tomato, however in 1967 in New Zealand this name was changed for the current name to prevent misunderstandings with the known garden tomato (Prohens and Nuez 2010). Nowadays the tamarillo plant is found in a lot of places around the world like Brazil, Australia, India, Southern Europe and others. It is the only member of its genus known to be self-compatible and autogamous which means that its flowers are self-pollinated (Bakshi and Jammu 2016). However wind and insects are needed for pollination of the flowers. If tamarillo tree is grown with limited wind and/or insects, like in a greenhouse, fruit set can be very low (Bakshi and Jammu 2016). In terms of edaphoclimatic conditions, tamarillo is a subtropical plant and grows in places with rainfall between 600 and 4000 mm and average annual temperatures of 15 up to 25 ºC and a soil pH of 5.0 to 8.5 (Prohens and Nuez 2010).

Tamarillo tree (Fig.1 A) grows fast and can have a height that goes until 5 meters. However this plant has a low life expectation living between 5 to 12 years. Its leaves are large, with 20 to 40 cm long and 20 to 35 cm wide, perennial and have a very strong fragrant smell. The flowers (Fig.1 B) have a pink white-corolla and form clusters that have 10 to 50 flowers producing 1 to 6 fruit. Tamarillo fruits are normally elliptical and measure 4 to 8 cm in length and 3 to 5 cm in width yet, round and elongated fruits also exists (Prohens and Nuez 2010).

Tamarillo fruits (Fig. 1 C) are known for being rich in vitamins such as vitamin A, E and C, minerals like potassium, magnesium, calcium and phosphorous, phenolic and carotenoids compounds and low in carbohydrates (4.7 g/100 g) and in calories (28 Kcal/100 g) (Bakshi and Jammu 2016). More, tamarillo fruit can be incorporated into jams, sauces, baby food or simply eaten raw as a dessert. They can be red, yellow and purple. The red one is the common type because of its bright color, being oval-shaped and the flesh orange-colored and weights between 50 to 80g (Bakshi and Jammu 2016). The yellow type has

yellow skin, is oval-shaped and its flesh is yellow too. The purple fruit has a dark red skin, its shape goes round to oval and the flesh is purple colored (Prohens and Nuez 2010).

Figure 1. *Solanum betaceum* Cav. **(A)** Tamarillo tree growing at the Botanical Garden of the University of Coimbra. **(B)** Flowers at different developmental stages. **(C)** Fruits. (Correia 2011)

Tamarillo can be propagated through conventional methods such as by seeds or cuttings. Tamarillo seeds germinate very easily but this method doesn't guarantee genetic uniformity because if seeds come from plantations with a variety of cultivars or seedlings, cross pollinations will happen and the seedlings can segregate for a series of features. So if the main purpose is the propagation of selected genotypes, tamarillo propagation by seeds is not adequate.

Tamarillo is one of many species in which numerous types of *in vitro* propagation have been reported (Correia and Canhoto 2012). Micropropagation can be defined as an *in vitro* method for the multiplication and development of new plants from plant tissues always under aseptic conditions (Bakshi and Jammu 2016). Axillary shoot proliferation on tamarillo was the first method tested (Cohen and Elliot 1979) and regeneration by organogenesis on leaf explants next (Obando et al. 1992). Then followed by the establishment of protocols for somatic embryogenesis induction and somatic embryo development, that has been effectively obtained from diverse explants like: roots, hypocotyls, cotyledons, mature zygotic embryos and leaves obtain by *in vitro* proliferating shoots (Guimarães et al. 1988; Canhoto et al. 2005; Correia and Canhoto 2012).

1.2. Somatic embryogenesis and callus induction in tamarillo

The high plasticity for cell differentiation is one central characteristic of plants, that are able to generate unorganized cell masses, such as callus or tumors, in response to stresses, such as wounding or pathogen infection (Ikeuchi et al., 2013). Under certain conditions, callus cells also undergo somatic embryogenesis (SE), a process in which embryos, that resembles zygotic embryos, are generated from somatic cells. The first descriptions of somatic embryogenesis came from experiences made by Reinert (1958) and Steward et al. (1958) with carrot cells in culture.

In an abridgment way, the SE procedure develops in two distinct phases: induction phase, in which differentiated somatic cells obtain embryogenic competence and proliferate as embryogenic cells, and expression phase where embryogenic cells exhibit their embryogenic competence and differentiate to develop somatic embryos. Somatic embryogenesis is a significant biotechnological tool in terms of rapid large-scale clone propagation (Correia and Canhoto 2012) and SE in tamarillo has been studied, and valuable protocols developed, representing an appropriate model to understand the cytological and molecular mechanisms involved in somatic embryo formation and development and is a morphogenic *in vitro* process that could be applied to plant cloning and genetic transformation (Correia and Canhoto 2012).

As described earlier, SE in tamarillo can be induced from different kinds of explants like internodes, young leaves, mature zygotic embryos and hypocotyl segments. Using zygotic embryos, these are cultured on MS (Murashige and Skoog, 1962) medium containing one auxin that can be: naphthaleneacetic acid (NAA), picloram or 2,4-

dichlorophenoxyacetic acid (2,4-D). Young leaves could be used too and they are cultured on MS induction medium containing 2,4-D or picloram. Culture media also include high levels of sucrose (9%), which increase embryo formation and SE induction efficiency up to 85% (Canhoto et al. 2005). Using NAA with zygotic embryos, a reduced callus is produced after 3-4 weeks in culture and 2-3 weeks later somatic embryos develop and this process has been called "direct somatic embryogenesis" (DSE). Here, proembryogenic competent cells already exist, so embryo formation results from a reprogramming of unorganized tissue that appears directly on the surface of organized tissues like leaves or zygotic embryos (Carman and Campbell 1990). However these calli induced and formed are not able to preserve their embryogenic potential so it lose ability to differentiate somatic embryos (Canhoto et al. 2005).

When zygotic embryos or young leaves are cultured in the presence of 2-4D or picloram, calluses with clusters of embryogenic cells were formed, which continued on proliferation. This type of induction has been referred as "indirect somatic embryogenesis" (ISE). Yet, in contrast with DSE, a major reprogramming of proliferating calli with embryogenic competence is necessary before embryo formation (Carman and Campbell 1990). When embryogenic calli are transferred to culture medium with low sucrose levels (2-3%) and without plant growth regulators, somatic embryos are formed and morphologically resemble those that are obtained by "one step somatic embryogenesis". Embryogenic callus can be maintained and subcultured in medium with 2,4-D or picloram for several months preserving the embryogenic potential. (Canhoto et al. 2005). In Figure 2 it is demonstrated the SE protocols here described.

Focusing on indirect somatic embryogenesis (ISE), after 8-12 weeks in culture, the explants used (leaves and zygotic embryos) can generate two types of distinguished calluses: a white, compact callus, with embryogenic ability when transferred to auxin-free conditions (embryogenic callus, EC) and a friable, mucilaginous callus with no regeneration ability (non-embryogenic callus, NEC). The embryogenic callus (Fig. 3 A) can be separated from the fast growing non-embryogenic callus (Fig. 3 B) (Correia et al. 2012), and subcultured singly, either in solidified or in liquid medium with the same composition of the induction medium. Thus, cell suspension cultures are possible to obtain from these different calluses types, particularly from the NEC lines, due to their friable characteristic (Alves et al. 2017).

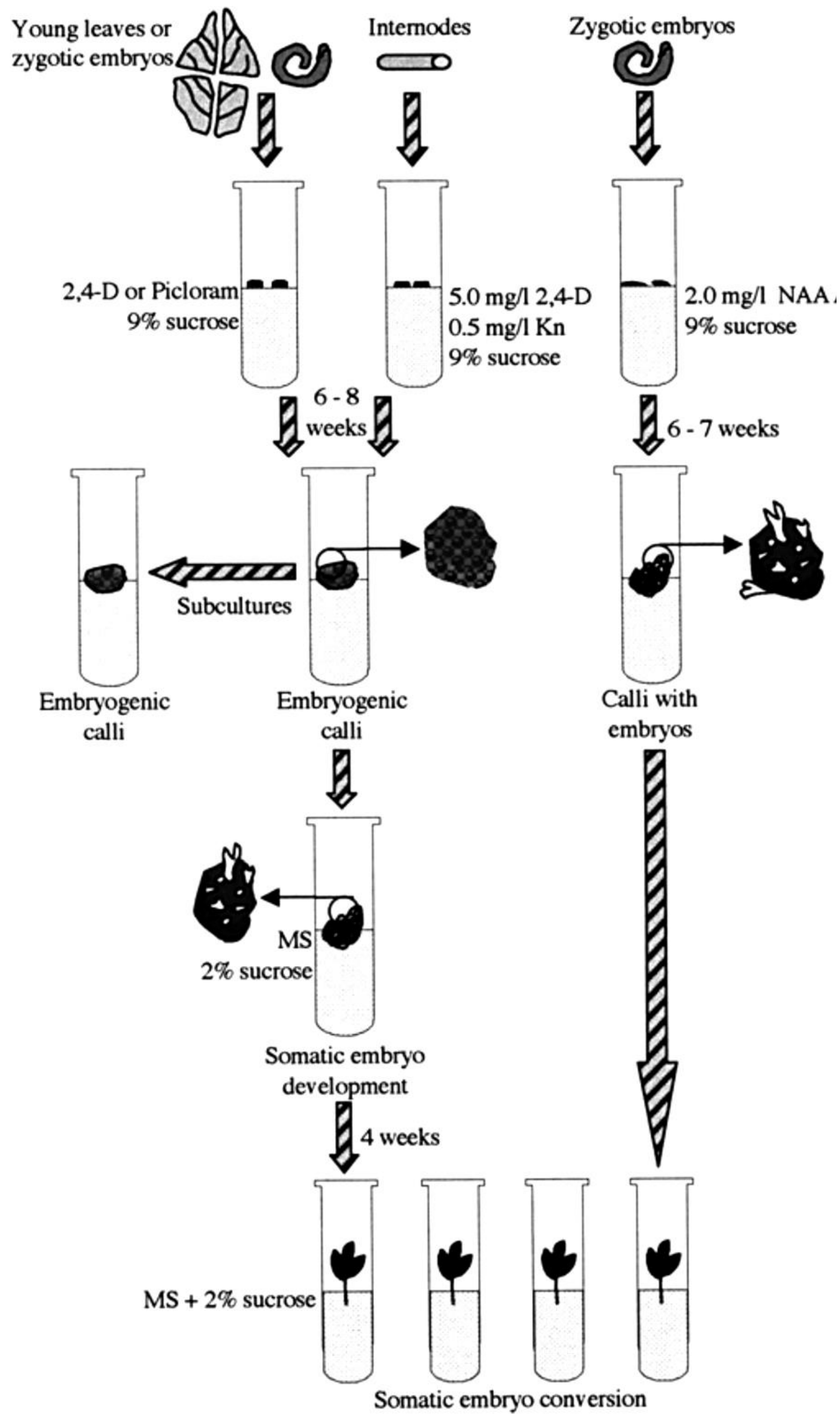

Figure 2. Schematic representation of the protocols for somatic embryogenesis induction in tamarillo. (Canhoto et al. 2005)

Figure 3. Macroscopic aspects of embryogenic (EC) and non-embryogenic (non-EC) calluses of *Solanum betaceum* obtained from leaf explants after a 12-wk culture period in the presence of picloram. (A) Embryogenic (ec) and non-EC callus (nec) on the same explant. (B) Non-embryogenic callus and (C) EC callus after 12 wk. of culture. (Alves et al. 2017)

1.3. Plant cell suspension cultures

First it is important to define what a plant cell suspension culture is: it is a sterile system starting with the insertion of friable callus fragments, under aseptic conditions, into an appropriate sterile liquid medium (Mustafa et al. 2011). Moreover, this aseptic system it is maintained under suitable conditions of agitation, light, temperature, aeration and other physical parameters (like carbon source and plant growth regulators type and concentration). Multiple culture media could be used, however the appropriated medium for an *in vitro* culture of, for example, plant tissues, plant cells or even protoplasts can be different depending on the species and/or genotype (Mustafa et al. 2011). The culture media most used for plant cell cultures are Murashige and Skoog (Murashige and Skoog 1962), Gamborg's B5 (Gamborg et al. 1968), Schenk and Hildebrandt medium (Sul and Korban 2004) and Linsmaier and Skoog (Linsmaier and Skoog 1965). In general, a culture medium presents microelements, macroelements, organic components, water and growth regulators (Mustafa et al. 2011). Microelements are essential in trace quantities for plant growth and development, comprising manganese, iodine, copper, cobalt, boron, molybdenum, iron and zinc (Smetanska 2008). Normally iron is added to the medium as iron sulphate and is conjugated with ethylenediaminetetraacetic acid (EDTA) allowing a slow and continuous release of iron into the medium (Smetanska 2008). Macroelements comprise elements needed in a large supply as potassium, nitrogen, phosphorous, calcium, magnesium and

sulfur (Smetanska 2008). Organic compounds include carbon sources (such as sugars) and vitamins. Plant cell cultures use simple sugars as carbon sources like sucrose, glucose, fructose, maltose and others. Commonly plant cells uptake glucose faster (after the hydrolysis of sucrose) than fructose (Mustafa et al. 2011). The optimal carbon source and its ideal concentration depends on the plant species (Smetanska 2008). In terms of vitamins the culture medium is usually enriched with thiamine (vitamin B1) and myo-inositol and these are very important for many biochemical reactions (Mustafa et al. 2011). Thought, other vitamins are often added to culture medium (Smetanska 2008). Plant growth regulators (PGRs) are very important once they induce calluses and stimulate the growth of many cell lines. Meanwhile each plant species and/or genotypes needs different types and concentrations of PGRs for callus induction, its growth and metabolites production. So it is required to select the most suitable growth regulators and define their ideal concentrations. The most common PGRs used are auxins and cytokinins and the kind and concentration of auxin or cytokinin or the auxin/cytokinin ratio need to be determined since they will modify growth and product formation in plant cell suspension cultures. Auxins are normally added to the culture medium at a concentration range between 0.1 to 50 µM and an intensification of auxin levels in the medium stimulates dedifferentiation of the cells, cell division, and callus formation and growth. Moreover, cytokinins are used in plant cell cultures at a concentration range of 0.1 to 10 µM, stimulating cell division and modulating callus initiation and growth (Smetanska 2008). Examples of auxins used are indole-3-acetic acid (IAA), a natural auxin, and synthetic auxins like 1-naphthaleneacetic acid (NAA), 2-4-dichlorophenoxyacetic acid (2,4-D) and 4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram). In terms of cytokinins, kinetin, zeatin and benzylaminopurine are the most used (Mustafa et al. 2011).

As described earlier, suspension cultures need proper physical growth conditions in which light, temperature, agitation, aeration and medium pH are included (Mustafa et al. 2011), influencing cell suspensions growth in several ways:

(i) Light: cell suspension cultures can be maintained in the dark, in periods of light and dark or in continuous light. Permanent exposure to light is easiest once full darkness situation is hard to accomplish, requiring special procedures for reproducible results. However a minimal exposure to light may induce different biosynthetic pathways (Mustafa et al. 2011);

(ii) Temperature: optimal cell suspension culture growth is accomplished in temperature ranges of 17 - 25 ºC (Rao and Ravishankar 2002);

(iii) Agitation: agitation or mixing is an important growth parameter since it promotes an improvement in the transference of nutrients from liquid and gaseous phases to the cells. Moreover, a better dispersion of air bubbles occurs, consequently providing a more effective oxygenation. On the other hand, high agitation levels lead to a high shear rate that reduce the mean aggregate size, but have a negative effect on cell viability. Therefore, cell suspension cultures are normally grown at very low agitation speeds (Chattopadhyay et al. 2002);

(iv) Oxygen and aeration: the necessity of oxygen levels in plant cell suspension cultures is relatively lower when compared with microbial cultures because in the first case the growth rates are lower. Moreover, in certain cases, high levels of oxygen are toxic to the cells' metabolic activities and might deprive nutrients like carbon dioxide (Chattopadhyay et al. 2002). Carbon dioxide is normally considered a crucial nutrient in the culture of plant cells and affects positively the cell growth by improving cell growth. The degree of air bubble dispersion, the culture mixing and the culture medium's capacity for oxygen affect appropriate aeration of the culture (Chattopadhyay et al. 2002).

(v) Medium pH: medium pH is usually adjusted between 5 and 6, avoiding extreme pH's. Throughout the culture growth there are alterations in hydrogen ion concentration and the medium pH increases during nitrate uptake and decreases during ammonia assimilation (Smetanska 2008).

Apart from this physical growth conditions, plant cell suspension cultures suffer from aggregation. Aggregation is a process in which plant cells, growing in liquid medium, form aggregates or clumps (Mustafa et al. 2011). Aggregation is common and mostly related to a failure of the cells to separate after division. On the other hand, aggregates or clumps are a direct consequence of the hydrodynamic stress associated to aeration and agitation factors. Also, the sensitivity of plant cells to this shear stress can be attributed to the physical characteristics of the suspended cells, like their size, the presence of thick cellulose based cell walls and the presence of large vacuoles. So, plant cells with larger sizes are capable of tolerating tensile strain but are sensitive to hydrodynamic shear stress. Besides leading to the formation of aggregates, shear stress can also have more consequences on plant cells, such as cell damage, that can be measured using a number of system responses like reduction in cell viability, release of intracellular compounds, alterations in morphology and changes in metabolism (Chattopadhyay et al. 2002). Nevertheless, a cell suspension culture with single cells is desirable to a combination of different sizes of cell aggregates as it is

easier to work with and a non-heterogeneous culture offers major reproducibility in investigates (Mustafa et al. 2011). However, as described too in Mustafa (2011), a certain degree of aggregates in strawberry cell suspension cultures demonstrated an improvement in anthocyanins production.

Cell suspension cultures are a suitable biotechnological model for the study and experimental analysis of cellular and molecular processes in a variety of plant species (Mulabagal and Tsay 2004). For this it is vital to analyze and measure cell growth resorting to several methods based on diverse parameters. The measurement of growth parameters becomes quite important once the objective is to obtain reproducible growth cycles of cell suspension cultures and to guarantee the reproducibility of production, whichever for research in the laboratory or in industrial scales. These growth parameters can be associated to the number of cells, the cell mass, protein content, cell viability or DNA content, between others (Mustafa et al. 2011). When cell suspension cultures are initiated from friable callus, the growth dynamic of these cells can be defined by a sigmoid curve. This curve is characterized by having a lag phase, corresponding to an adaptation period of the cells to new medium, an exponential and a linear phases, in agreement with a high rate of cell division that results in the suspension culture growth, and a stationary phase where the rate of cellular divisions is progressively reduced (Silveira et al. 2004).

The first reference to plant cells liquid cultures was in the 1950s (as reviewed by De Vijlder et al., 2015). As already mentioned, cell suspension cultures are important tools to analyze specific and complex cellular and biochemical processes like cell differentiation, cycle progression and biochemical synthesis of secondary metabolites (De Vijlder et al. 2015). Additionally they are also used as living “factories” in terms of the production of recombinant proteins, comprising antibodies and cytokines (De Vijlder et al. 2015). Several plant species have been used for establishment and proliferation of cell suspension cultures, alternating from model systems like *Arabidopsis thaliana* (Borderies et al. 2003), *Catharantus* (Zenk et al. 1977) and *Taxus* (Yukimune et al. 1996), to imperative monocotyledon or dicotyledon crop species like rice (Toriyama and Hinata 1985), alfalfa (Kao and Michayluk 1981) and tobacco (Ericson and Alfinito 1984).

In vitro dedifferentiated plant cell suspension cultures offer the advantage of a not complex model system for the study of plants (Mustafa et al. 2011), when compared to the use of whole plants. In whole plants there is the impact of pests and diseases and the performance of the cultured plant is reliant on the climate, soil quality, season, day length and weather. Moreover, cell suspension cultures don’t have the risk of contamination with

mycotoxins, pesticides and herbicides (Kwon et al. 2003). Besides, plant cells are biosynthetically totipotent, which incomes that every single cell in the culture holds complete information and therefore is capable to produce the sort of products and metabolites found in the parent plant (Rao and Ravishankar 2002). Maybe the most important gain of plant cell suspension cultures over whole plant is the much simpler procedure for product isolation, particularly if the product/metabolite of interest is secreted into the culture medium (Hellwig et al. 2004).

1.3.1. Plant cell suspension cultures protein production

Cell suspension cultures represent a practical system allowing the study of molecular and physiological occurrences in homogeneous single cells and, moreover, they have been used in molecular studies for monocotyledons and dicotyledons species, as referred earlier (Liu et al. 2013). Proteomics is the study of proteins and their functions in a cell, tissue, organ and organism (Agrawal et al. 2011). Proteome study and analysis normally represents an extent of valuable information capable of describing the state of a biological entity (De Vijlder et al. 2015). The proteomics subject has become quite significant in plant sciences due to numerous studies on important crops like maize (Riccardi et al. 2002), rice (Rakwal and Agrawal 2003) and *Medicago truncatula* (Watson et al. 2003). Plant cells secrete an assortment of proteins into the apoplast or bind them into the cell wall, and the apoplast function includes growth regulation, tissue structure, defense against biotic and abiotic stress factors, osmotic homeostasis, transport, gas exchange and cell adhesion (Lippmann et al. 2009). For example, in *Arabidopsis thaliana*, 400 proteins were recognized as cell wall components, corresponding to almost 25 % of the total assessed extracellular proteins (Chen et al. 2009). Additionally, accordingly to classical studies of plant proteomics, a noteworthy collection of plant proteins were localized in specific subcellular compartments, such as membranes or organelles (Tanaka et al. 2004). Furthermore, proteins produced in cell suspension cultures might be signaled to the secretory pathway, reaching the apoplast, from where they will diffuse over the cell wall to the culture liquid medium. However, depending on the size of the proteins and/or their physicochemical properties they can be trapped in the cell wall matrix or secreted into the medium. Generally, proteins of less than 30 kDa have the tendency to be released into the liquid medium while bigger proteins are retained (Hellwig et al. 2004).

Suspension cultures of cells with regeneration ability, such as in the case of embryogenic competent cells induced by somatic embryogenesis, has become an important system to study the molecular mechanisms that are on the genesis of this biotechnological process of plant development (Mulabagal and Tsay 2004). Carrot cells suspension cultures were one of the first models to be explored and studied regarding the understanding of the process of somatic embryogenesis from embryogenic cell clusters (Komamine et al. 1992). Several studies had been made in order to define somatic embryogenesis at the molecular level, using proteomics and transcriptomics methodologies, in many plant species (Marsoni et al., 2008; Correia et al. 2012). A variety of proteins involved in a numerous of somatic embryogenesis cellular processes have been recognized in different woody species, including tamarillo (Correia et al. 2012; Alves et al. 2017), that goes from stress response, storage proteins, cell proliferation and cell wall metabolism, until metabolism and energy state, signal transduction and protein synthesis and processing (Guan et al. 2016).

In tamarillo, the proteomics analysis made were initially concentrated on intracellular biochemistry of embryogenic and non-embryogenic cells (Correia et al., 2012) and later on tamarillo cell suspension cultures to compare extracellular protein profiles in embryogenic and non-embryogenic calluses (Alves et al. 2017).

1.3.1. Elicitation of products production

Some biotechnological approaches have been made and experimented for the enhancing of products production by plants, either by media modification, screening of high yielding cell lines, precursor feeding, large scale cultivation in bioreactor systems, hairy root cultures, biotransformation processes, elicitation and others (Ajay 2007). The elicitation strategy was studied and investigated in the present work. Elicitation is the process in which the induced or enriched synthesis of products occurs by the addition of small amounts of molecules or compounds named elicitors (Ajay 2007). Elicitors are substances added in trace concentrations to a living cell system that promotes the upgrading of specific products (Ajay 2007). Plants display a wide range of defense mechanisms against pathogen attack which can be constitutive or induce defense strategies. In the last case, plants activate these defense cascades in the presence of elicitors (Angelova et al. 2006).

Elicitors (Fig. 4) can be categorized accordingly to their “nature” like abiotic or biotic elicitors, or on the genesis of their “origin” like endogenous and exogenous elicitors (Ajay 2007). As the term indicates, abiotic elicitors are the substances that have a non-biological origin, from which inorganic salts, metal ions, electric current, hormones and high pH are examples (Weathers et al. 2010). Biotic elicitors have a biological origin, including polysaccharides derived from plant cell walls, like pectin or cellulose, or from microorganisms, such as chitin or glucans, G-proteins or glycoproteins or intracellular proteins (activate or inactivate ion channels or enzymes) and yeast extract and casein hydrolyzed. On the other hand, elicitors defined as exogenous elicitors are compounds that have their origin outside the cell (polysaccharides, polyamines and fatty acids) whereas endogenous elicitors are compounds created inside the cell (galacturonide or hepta-β-glucosides) (Ajay 2007).

Plant cells or organs are a chemical factory, so with a greater size of the factory comes a higher amount of product that can be formed. Though to obtain high amounts of products, high quantities of plant biomass are commonly necessary (Weathers et al. 2010). It has been described that some abiotic and biotic elicitors, like yeast extract, casein hydrolyzed, chitosan, jasmonic acid and salicylic acid, can be supplemented to plant cell suspensions cultures in order to enhance the biomass yield (Trong et al. 2017). Certainly with the optimization of the biomass callus growth the production of compounds will be superior (Weathers et al. 2010).

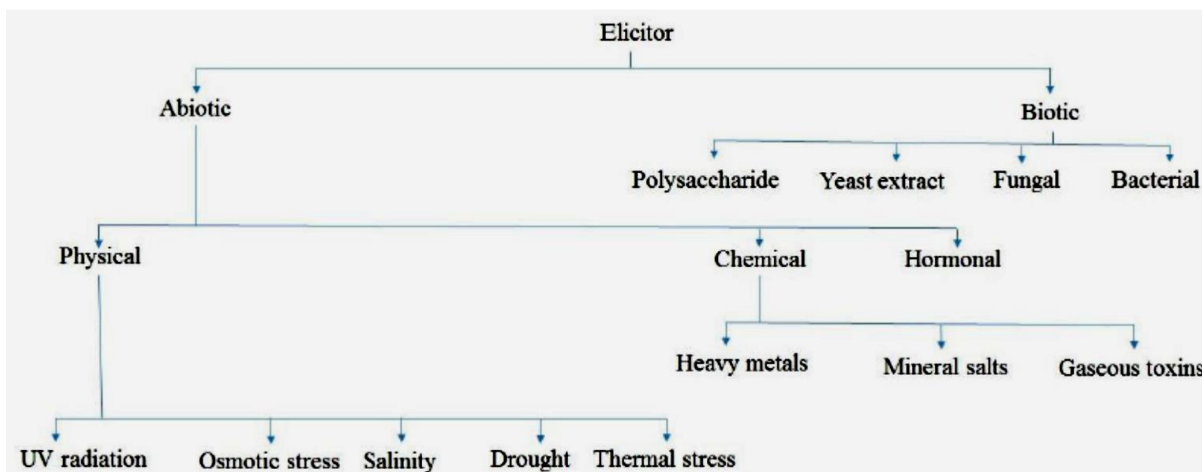

Figure 4. Elicitors classification centered on their nature (Naik et al., 2016).

Plant cells or organs are a chemical factory, so with a greater size of the factory comes a higher amount of product that can be formed. Though to obtain high amounts of products, high quantities of plant biomass are commonly necessary (Weathers et al. 2010). It has been described that some abiotic and biotic elicitors, like yeast extract, casein hydrolyzed, chitosan, jasmonic acid and salicylic acid (abiotic elicitors), can be supplemented to plant cell suspensions cultures in order to enhance the biomass yield (Trong et al. 2017). Certainly with the optimization of the biomass callus growth the production of compounds will be superior (Weathers et al., 2010).

In the present work two different biotic elicitors were tested: yeast extract and casein hydrolyzed. Yeast extract has demonstrated a positive effect when added to suspension cultures: yeast extract enthused ethylene biosynthesis in *Solanum lycopersicum* (tomato) (Felix et al. 1991) and with *Panax ginseng* cultures led to an increase in biomass and in the production of secondary metabolites (Trong et al. 2017). In terms of casein hydrolyzed, several studies using this elicitor have also been made. Using 0.5 g/L of casein hydrolyzed led to an increase of stevioside metabolite in *Stevia rebaudiana* suspension cultures and when added to calluses cultures of *S. rebaudiana*, it also intensified the accumulation of this metabolite (Bayraktar et al. 2016).

The use of elicitors in plant cell suspension cultures may cause several biological effects like: an increase in the production of secondary metabolites, phytoalexin biosynthesis, intensification in the activity of defense enzymes and differential gene expression (Namdeo 2007). The exact mechanism behind elicitation is still unclear. Some investigators emphasize the rapid modifications in protein phosphorylation configurations and protein kinase activation as a mechanism of elicitation (Namdeo 2007). Another group of researcher's hypothesis is that the accumulation of defense-related proteins pathogenesis related proteins like chitinases and glucanases contribute to the release of signaling oligomers (endogenous elicitors), protease inhibitors and glycoproteins (Benhamou 1996). Or even that the elicitation process lead to a transcriptional activation of defense response genes (Namdeo 2007).

The way of action of elicitors relies on numerous factors like: (i) elicitor concentration, (ii) elicitor selectivity, (iii) age of culture, (iv) duration of the contact with the elicitor, (v) cell line and others. For example, elicitor concentration has a very important part in elicitation mechanism, once high dosage of elicitor may lead to hypersensitive response resulting in cell death. So, an adequate elicitor concentration is required for an effective induction (Namdeo 2007)

1.4. Bioreactors

Plant cell cultures are used to produce appreciated natural products like pharmaceuticals, flavors and fragrances, and reasonable chemicals (Sajc et al., 2000). Bioreactors are a suitable model for the large-scale production of important products in the pharmaceutical industry, food industry and cosmetics (Eibl et al. 2008). Some examples that are produced in large-scale are shikonin, ginsenosides and berberine, all manufactured in Japan (Sajc et al. 2000). Moreover, the anti-cancer drug paclitaxel from *Taxus* cells has already some strategies to improve it production like the use of methyl jasmonate elicitor (Yukimune et al. 1996).

Bioreactors systems are defined in a biochemical perspective as self-contained, liquid sterile environments which are designed for large-scale intensive culture, monitoring and controlling several conditions (aeration, agitation, dissolved oxygen, temperature and pH) (Paek et al. 2005).

The large-scale plant cell and tissue cultures have been considered as a valuable substitute source of biochemicals in the past 40 years (Sajc et al. 2000). The study of plant cells and tissues in terms of growth, development and metabolism have been made to improve the understanding of plant cell biology and tissue physiology, as well as to develop norms to bioprocess design (Sajc et al. 2000).

There are three central classes of culture system in bioreactors: (i) those that produce biomass (as cells or organogenic or embryogenic masses, shoots or roots); (ii) those which produce metabolites and enzymes; and (iii) those that are used for biotransformation of exogenously added metabolites (that could be precursors in a metabolic pathway) (Paek et al. 2005).

These systems offer diverse advantages as controlled source of biochemical supplies independent of plant availability (like pests, politics and cultivation season) and well distinct structures capable of produce higher yields (Paek et al. 2005).

Moreover, plant biotechnology needs a deep research in areas like plant physiology, cell and molecular biology, pharmacology, chemistry, toxicology and chemical engineering (Sajc et al., 2000). This search allow the estimate of: (i) tissue morphology and composition, (ii) flow and mass transfer circumstances in the bioreactor, (iii) kinetics of cell growth and product formation, (iv) genetic stability of high-producing cellular lines, (v) consequences of bioreactor construction on downstream processes and (vi) the prospective for process scale-up (Sajc et al., 2000). However, the in situ product extraction, the use of cell-polymer

constructs, addition of different precursors, elicitors, and medium altercation approaches can increase the plant tissue culture, inside the bioreactor, more than any of the earlier aspects on their one (Sajc et al. 2000).

1.5. Objectives

In tamarillo, SE is well described and supported by several protocols (Canhoto et al. 2005; Correia and Canhoto, 2012). As described earlier, in tamarillo ISE it is possible to obtain and distinguish two kinds of calluses, embryogenic and non-embryogenic callus (EC and NEC, respectively). In this work the main focus will fall back on tamarillo NEC, using two different non-embryogenic cellular lines. Also, cell suspension cultures are another significant biotechnological instrument, previously developed for tamarillo (Alves et al. 2017), which allows that plant cells can be investigated and further characterized in terms of biochemical processes and metabolites production and even be used to produce specific proteins.

The main goal of this work is to characterize and optimize tamarillo cellular lines cultures using a bioreactor system in terms of:

a) Cellular Growth: growth curves and cell number counting;
b) Metabolites production: protein quantification, mass quantifications, protein profiles and proteolytic profiles;
c) Elicitors influence: protein quantification, mass quantifications, protein profiles and proteolytic profiles.

2. Materials and Methods

2.1. Plant material and culture conditions

2.1.1. Tamarillo calluses *in vitro* induction and sub-cultures

The tamarillo calluses used in this study were previously obtained by somatic embryogenesis induction using young leaves from tamarillo clones, according to the established protocol (Correia and Canhoto, 2012). The induction medium used was MS (Murashige and Skoog, 1962) basal medium (Duchefa Biochimie ©) supplemented with 9% sucrose (w/v), a synthetic auxin, picloram, in a concentration of 5 mg/L and 2.5 g/L of Phytagel (Sigma). The pH of the medium was adjusted to 5.7 (with HCL or KOH) before autoclaving at 121°C for 20 min. Two types of tamarillo callus lines, induced from two different genotypes were used, once two kinds of leaf explants were placed on the induction medium and maintained in the dark in a growth chamber at a 24°C ± 1°C during 8-12 weeks. After the induction phase, proliferated masses of non-embryogenic cells were isolated and monthly sub-cultivated in test tubes with 12 mL of the same jellified medium with 9 % (w/v) sucrose and 5 mg/mL of picloram. Non-embryogenic line 1 (NEL 1) and non-embryogenic line 2 (NEL 2) are the non-embryogenic cellular lines arising from the two tamarillo genotypes used.

2.1.2. Cell suspension cultures establishment and growth

In order to study NEL 1 and NEL 2 in terms of products production, a system similar to a laboratory scale bioreactor was carried out. The cellular lines were grown in two liquid MS media: one with 3% (w/v) of sucrose and 5 mg/L of picloram (referred as M3) and the other with 9% of sucrose (w/v) and 5 mg/L of picloram (referred as M9). A number of three replicates per condition was accomplished, giving a total of 6 samples for each cell line. A *ratio* of 500 mg of callus per 250 mL of MS liquid medium was used, by transferring masses of proliferating callus (after 4 weeks of sub-culture) from the jellified medium previously described to the liquid culture media in 500 mL erlenmeyers. This process was performed

in a horizontal laminar flow chamber in aseptic conditions. The suspension cultures were maintained in an orbital shaker at 100 rpm in the dark at 24ºC ± 1ºC, for two weeks.

2.1.3. Cell suspension cultures growth with elicitors

The hypothesis that the products yield and the final callus biomass could be enhanced was tested subjecting the cells to the presence of biotic elicitors. Two kinds of biotic elicitors were evaluated: casein hydrolyzed and yeast extract (Sigma). Casein hydrolyzed (CH) was added to the culture medium in two different final concentrations: 0.5 g/L and 1.5 g/L. Moreover, this biotic elicitor was weighty, dissolved in distilled water, filter sterilized and added to the liquid culture medium after pH adjustment and sterilization. The same final concentrations were used to yeast extract: 0.5 g/L and 1.5 g/L. Once, yeast extract (YE) was dissolved in distilled water and placed in a glass flask to further sterilization. In this case, the liquid culture medium was supplemented, at aseptic conditions, with yeast extract during cellular growth exponential phase and not at the beginning as for casein hydrolyzed. So NEL 1 and NEL 2 were grown in liquid medium with this two biotic elicitors, at the final concentrations referred above, and without elicitors (standard/control). In this phase, each cell line was grown with five conditions: standard liquid culture medium (SM), liquid medium supplemented with 0.5 g/L and 1.5 g/L of casein hydrolyzed (CH [1] and CH [2], respectively) and liquid medium supplemented with 0.5 g/L and 1.5 g/L of yeast extract (YE [1] and YE [2], respectively). Here, SM had a sucrose concentration of 3% (w/v) (data presented at results chapter). A number of three replicates per medium was accomplished, giving a total of 15 samples for each cell line. Again, a ratio of 500 mg of callus per 250 mL of MS liquid medium was used, following the same procedure as referred above.

2.1.4. - Growth measurements

Aliquots of 1 mL from all samples were took during cell growth to be analyzed in terms of optical density and total number of cells. The first growth evaluations (optical density and number of cells) were made 1 hour after growth initiation. Optical density of the suspension cultures was evaluated by measuring their absorbance at 630 nm in quartz

cuvettes in a BIOCHROM ULTROSPEC II spectrophotometer. During this measurements all of the samples were homogenized to avoid the sedimentation of cells on the base of the cuvettes. The number of cells evaluation was carried out using a SEDGEWICK RAFTER counting chamber with 1 mL of the suspension cultures and then visualized on an optic microscope.

The total number of cells was obtained by applying the next equation (1):

(1) Total Number of cells=Number of cells/mL*Suspension Cultures Volume

With the optical density measurements a growth curve of the cells was drawn for the different media in function of the cultures time until an optimum value of optical density was reached and the cultures growth finished. The accounting of the total number of cells was necessary to substantiate the growth curves and to relate the increase of biomass with the cellular division.

2.2. Protein analysis

Attained the ideal optical density and subsconsequently finished the growth of NEL 1 and NEL 2 cell lines the protein produced was analyzed. First the cell suspensions cultures were filtered under vacuum to separate the liquid medium from the cells. In order to obtain an efficient separation of the cells and the culture medium, a 0.45 μm Sartorius Stedim Biotech® filter was used. The cells retained in the filter were weighted and the liquid medium stored at - 4 ºC.

2.2.1. Intracellular protein

Weighted the cells, it was important to optimize the protein extraction method from the cells. Since the first method used was poorly efficient (mechanic treatment with a plastic piston – data at results chapter), three other protein extraction methods were tested:

i. Mechanic treatment with glass beads: 5 g of glass beads (Sigma) with 425-600 microns were placed in a 50 mL falcon tube. First, the beads were washed 5-6 times with distilled water and further centrifuged to remove the water. About 7mL of sodium phosphate buffer 0.05 M pH 7 were added to the glass beads and then the cells recovered were added too. This system glass beads-cells-buffer was submitted to vortex several times until full homogenization. Finally the system was centrifuged during 10 minutes, 14000 rpm, and the supernatant recovered.
ii. Mechanic treatment with liquid nitrogen: the recovered cells were placed in a mortar and liquid nitrogen was added. The cells were grinded until a fine powder was obtained. Next, 5 mL of sodium phosphate buffer 0.05 M pH 7 were added and the samples were homogenized and collected to falcon tubes. To finish, samples were centrifuged at 14000 rpm during 15 minutes and the supernatant was recovered.
iii. Enzymatic treatment with pectinase: this extraction method was made after the cell's filtration. The aim of this process was to weak the cells' walls in order to facilitate cell membrane lysis with further filtration and centrifugation. First a pectinase solution was prepared with 0.15 % (w/v) pectinase dissolved in 10 mL of M3 (MS medium with 3% w/v sucrose) and the pH adjusted to 5.5. After this, 1 mL of this solution was placed in a water bath at 55 ^{0}C during 10 min to activate the enzyme. Subsequently this 1 mL was added to the cells recovered after filtration from the suspension cultures and an enzymatic digestion with the pectinase solution was carried out for 4 hours at 37 ^{0}C and 100 rpm. After this step, the digestion solution was filtrated and the cells recovered. Callus mass was weighted and then 5 mL of sodium phosphate buffer 0.05 M pH 7 was added to the cells. Samples were centrifuged at 14000 rpm during 15 minutes and the supernatant was recovered.

After the recovery of supernatants obtained from all the samples total protein was quantified. For this the Bio-Rad Protein Assay protocol was used, more specifically the Microassay Procedure for Microtier Plates using a 96-well microplate, as described by the manufacturer. A calibration curve was produced using concentrations of bovine serum

albumin (BSA) between 8 and 80 μg/mL. All the samples and calibration curve concentrations were analyzed and quantified in triplicate at 595 nm using a SPECTRAmax PLUS 384 spectrophotometer.

2.2.2. Extracellular Protein

The liquid media stored at - 4 ºC were manipulated, in order to analyze the presence of extracellular proteins, by performing a dialysis and further a lyophilization. To make the dialysis, liquid medium was retained on dialysis membranes and these in turn were placed inside a 1000 mL plastic glass containing sodium phosphate buffer 0.05 M pH 7. Dialysis were maintained overnight at - 4 ºC. The samples from dialysis were frozen at - 80 ºC and then placed in a lyophilizer during 3 days. Lyophilized samples were solubilized in 5 mL of sodium phosphate buffer 0.05 M pH 7.

After the collecting of lyophilized samples it was necessary to quantify the amount of total protein present in the samples. For this the Bio-Rad Protein Assay protocol was used, more specifically Microassay Procedure for Microtier Plates using a 96-well microplate. A calibration curve was carried out using concentrations of BSA between 8 and 80 μg/mL. All the samples and calibration curve concentrations were analyzed and quantified in triplicate at 595 nm using a SPECTRAmax PLUS 384 spectrophotometer.

2.2.3 Protein profiles

2.2.3.1 SDS-PAGE Profiles

In order to obtain protein profiles from intra and extracellular (lyophilized samples) samples a protein concentration protocol with methanol and chloroform was performed. For this 200 μL of protein sample were added to a 2 mL eppendorf. To this volume of protein sample, the following were added:

1. 800 μL of methanol, with further vortex;
2. 200 μL of chloroform, with further vortex;

3. 600 μL of distilled water, with further vortex.

This mixture was then centrifuged at 14000 rpm during 5 minutes. At the top was the water phase, in the interphase was the protein and in the bottom was the chloroform phase. The water layer was removed and 800 μL of methanol added followed by vortex. Finally this was centrifuged again at 14000 rpm during 5 minutes and the supernatant (methanol) was removed. A *pellet* was observed on the bottom of the eppendorf and was left to dry at room temperature. Then, a Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out. Denaturing buffer (125 mM Tris-HCL, pH 6.8; 100 mM glycine; 40% v/v glycerol; 4% w/v SDS; 200 mM DDT; 0.001% w/v bromophenol blue) was added to the samples and then incubated in an eppendorf thermoblock at 90 ºC during 10 minutes. Next the denatured samples were loaded into a 12.5% polyacrylamide gel (2.4 mL of mili-Q water, 1.5 M Tris pH 8.8, 40% acrylamide, 20% SDS, 10% AMPS and 7 μL of TEMED) and then submitted to a voltage of 120 V for an hour and half at room temperature. After this, the gels were stained in a solution of 0.25% (w/v) Coomassie Brilliant blue R250 with 45% (v/v) methanol and 5% (v/v) acetic acid during 15 minutes. The gels were transferred from this solution to a washing solution containing 25% (v/v) methanol and 5% (v/v) acetic acid. When no protein staining was visible with Comassie blue, the gels were stained using silver nitrate. To stain the gels with silver nitrate first it was necessary to pass the SDS-PAGE gels to a 50% (v/v) methanol solution during 10 minutes in order to wash them; this step was repeated again but with a 30% (w/v) methanol solution; after this, the gels were transferred and incubated sodium thiosulfate (0.2 g/L) during 1 minute; Next, sodium thiosulfate was removed and the gels were washed twice with distilled water during 20 minutes; After the washing of the gels, a silver nitrate solution (2.0 g/L) was prepared and was added to the gels; these were maintained in this solution during 20 minutes (incubation); This silver nitrate solution was removed and the gels were rapidly washed with distilled water; Furthermore, a revelation solution was prepared: 37% (v/v) of formaldehyde (0.7 mL/L), 30 g/L (w/v) of sodium carbonate and 10 mg/L (w/v) of sodium thiosulfate; After this solution was added to the gels; This solution was maintained in contact with gels just until the intended coloring of gels; Finally, this coloring process was stopped with the addition of Tris (50 g/L) and 2.5% (v/v) of acetic acid during 1 minute; The gels were stored in distilled water.

2.2.3.2 Zymography

Intracellular samples were submitted to a zymography in order to identify proteolytic enzymes. A gel with 12.5% polyacrylamide was made, in the same conditions referred above, but with the inclusion of gelatin, proteolytic enzymes substrate, to a final concentration of 0.1%. Zymography buffer without reducing agents (125 mM Tris-HCL pH 8.8; 15% sucrose (w/v); 2.5% SDS (w/v) and 0.001% bromophenol blue) was added to the samples. Next these samples were loaded into the 12.5% polyacrylamide gel and a voltage of 100 V was applied for an hour and half at room temperature. After this, the gel was collected and washed in a renaturation buffer (30 mM phosphate buffer pH 6.0 and 1% Triton X-100) during 2 hours and then incubated overnight in a warm house at 37ºC in the samples buffer (sodium phosphate buffer 0.05 M pH 7).

2.2.4. Proteolytic profiles

The proteolytic profiles were essayed using enzymatic activity assays. The first group of enzymatic substrates used for the presence of proteases had a fluorogenic group in it C-terminal: amino methylcoumarine (AMC). These substrates were amino acids: arginine (L-Arginine-4-methylcoumaryl-7-amide: Arg-AMC), methionine (L-Methionine-4-methylcoumaryl-7-amide: Met-AMC), phenylalanine (L-Phenylalanine-4-methylcoumaryl-7-amide: Phe-AMC), lysine (L-Lysine-4-methylcoumaryl-7-amide: Lys-AMC), alanine (L-Alanine-4-methylcoumaryl-7-amide: Ala-AMC), leucine (L-Leucine-4-methylcoumaryl-7-amide: Met-AMC) and complex substrates: Glycyl-L-Proline-7-amide-4-methylcoumaryl (Gly-Pro-AMC), T-Butyloxycarbonyl-L-Valyl-L-Prolyl-L-Arginine-4-methylcoumaryl-7-amide (Boc-Val-Pro-Arg-AMC) and Succinyl-L-Alanyl-L-Alanyl-L-Prolyl-L-Phenylalanine-4-methylcoumaryl-7-amide (Suc-Ala-Ala-Pro-Phe-AMC).

Reactions took place in a 96-well microplate for fluorescence assays adding 2 µL of substrate to 100 µL of sample (50 µL of sodium phosphate buffer 0.05 M pH 7 and 50 µL of protein sample) using a SpectraMAX-GemiNI microwell fluorescent reader at 37º C with an excitation and emission wavelength of 380 and 460 nm, respectively, during 40 minutes. Furthermore another group of substrates were tested and in this case the fluorogenic group was methylumbelliferyl (MU). The substrates tested were: 4-Methylumbelliferyl-β-D-

glucopyranoside (MU-G), 4-Methylumbelliferyl-acettyl-β-D-glucosaminide (MU-NAG), 4-Methylumbelliferyl-acettyl-β-D-phosphate (MU-P) and 4-Methylumbelliferyl-acettyl-β-D-glucosaminidase (MU-NAG). MU-G, MU-NAG and MU-C are enzymatic substrates directed to glycoside hydrolases and MU-P to alkaline phosphatases. Once again the reaction took place in a spectraMAX-GemiNI microwell fluorescent reader at 37º C during 40 minutes but in this case the excitation and emission wavelength were 365 and 460 nm, respectively. All the results obtained with the two groups of substrates were in relative fluorescent units (RFU) per second and then were converted to pmol AMC/min/μg of protein.

Additionally to detect the main classes of proteases in the samples, an assay with 6 proteases inhibitors was carried out: Pepstatin A (aspartyl protease inhibitor) (A), EDTA (metalloprotease inhibitor) (B), TPCK (Tosyl phenylalanyl chloromethyl ketone - chymotrypsin and cysteine protease inhibitor) (C), TLCK (Tosyl-L-lysil-chloromethane hydrochloride - trypsine-like serine protease inhibitor) (D), E-64 [(Guanidinobutyl)amino)-4-methyl-1-oxopentan-2 yl)carbamoyl)cyclopropanecarboxylic acid - cysteine protease inhibitor] (E) and Pefabloc (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride - chymotrypsin-like serine protease inhibitor) (F). This assay was performed gathering the inhibitors in cocktails. Instead of adding just one enzymatic inhibitor to the samples, cocktails of inhibitors were performed. Cocktail 1 (C1) had all of the inhibitors and 6 more cocktails were made in which one inhibitor was removed:

i.	**C1: A+B+C+D+E+F**
ii.	**C2: B+C+D+E+F (A removed)**
iii.	**C3: A+C+D+E+F (B removed)**
iv.	**C4: A+B+D+E+F (C removed)**
v.	**C5: A+B+C+E+F (D removed)**
vi.	**C6: A+B+C+D+F (E removed)**
vii.	**C7: A+B+C+D+E (F removed)**

Inhibitors (final concentration of 1mM) was incubated with 100 μL of sample (50 μL of sodium phosphate buffer 0.05M pH 7 and 50 μL of protein sample) during 10 min at room temperature. After this, 2 μL of Gly-Pro-AMC substrate was added to the mixture to initiate the reaction. The reaction took place in a spectraMAX-GemiNI microwell fluorescent reader at 37º C during 40 minutes with an excitation and emission wavelength of 380 and 460 nm. The results were obtained in relative fluorescent units (RFU) per second and then were

converted to % of residual activity in relation with a control for each sample (sample without inhibitors).

2.3. Statistical analysis

Each analysis was carried out with 3 biological replicates (n=3). In first place it was important to evaluate the similarity between samples variances and this was accomplished using Brown-Forsythe test ($p<0.05$), for a set of data organized in more than two groups, and F test ($p<0.01$), for a set of data grouped in two groups.

For data groups bigger than two, if the variances were uniform, the data was analyzed with one-way analysis of variance (ANOVA) and the means were compared by Tukey test ($p<0.05$) or by Dunnett's multiple comparison test ($p<0.05$) (depending if the comparison was between samples or against a control sample, respectively). If the variances weren't homogenous, the non-parametric Kruskal-Wallis one-way analysis of variance was used and the means were compared by Dunn's multiple comparison test ($p<0.05$).

For comparisons between two groups, if the variances were homogeneous, the data was analyzed with unpaired t test ($p<0.05$). If the variances weren't similar, Welch's unequal variances t test ($p<0.05$).

3. Results and Discussion

3.1. Cell suspension cultures growth

The two cellular lines arising from tamarillo ISE are NEL 1 and NEL 2. These two cellular lines were previously established *in vitro* in solid MS medium with a sucrose concentration of 9% (w/v) and it was important to evaluate which sucrose concentration was better: 9% (w/v), concentration used to maintain non-embryogenic cellular lines *in vitro*, or a lowest concentration of 3% (w/v). So, 500 mg (FW) of each callus were placed inside flasks containing MS medium: three replicas with 9% (w/v) sucrose (M9) and another three with 3% (w/v) sucrose (M3). Various parameters were studied, starting with the appreciation of optical density, that allowed the construction of growth curves for these two cellular lines in order to sucrose concentration applied (Figure 5).

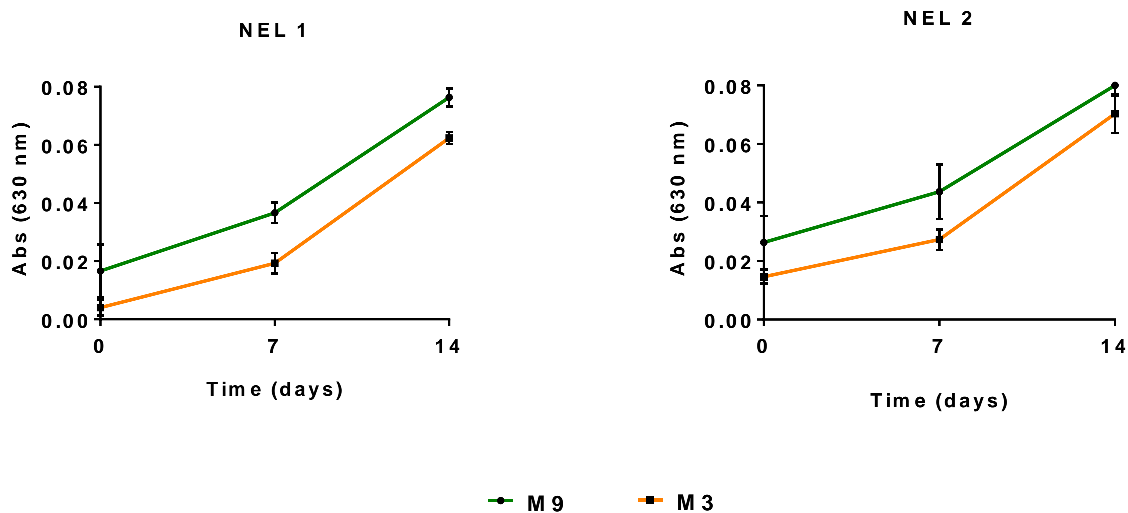

Figure 5. NEL 1 and NEL 2 growth evaluation. Growth curves distinguished by sucrose concentration: M9 - 9% (w/v) sucrose; M3 - 3% (w/v) sucrose. Data are represented as mean ± SD (n=3).

NEL 1 and NEL 2 growth curves, presented in figure 5, are quite similar. During the cellular growth, performed for 14 days, both lines demonstrated higher optical density values with 90 g/L of sucrose in liquid medium than 30 g/L of sucrose. Both graphs presented an initial phase with lowest optical values (first day until day 7) and a second phase with an

increase in those values (since day 7 until day 14). Accordingly to the optical density values, since day 7, there was a higher increase in cultures cellular growth. Now, comparing NEL 1 and NEL 2, the non-embryogenic cellular line 2 in M9 and in M3 showed major optical values than NEL 1 M9 and NEL 1 M3.

Optical density is a growth parameter capable of measuring cellular density. High cellular densities/biomass correspond to high optical density values. Sucrose, a carbon source, is an essential substrate to the production of energy used in cell's primary and secondary metabolism (Fazal et al., 2015). Though, increase the culture medium levels of sucrose has two different effects on cell suspension cultures: the first effect is the increase of osmotic pressure, altering the cellular environment, and the second one is the intensification of accessible carbohydrate sources as substrates in the liquid medium (Kim et al. 2001). On the other hand, sucrose is an important nutrient to the plant cell growth. As figure 5 demonstrates, liquid medium with 9% (w/v) sucrose (M9) appears to affect more positively the cells growth than 3% (w/v) sucrose (M3). Several studies have been made in order to relate the cell growth with the initial sucrose concentration supply. However, in these studies an elevate sucrose concentration demonstrated a decrease in cell growth. In cultured *Daucus carota* cells (carrot), sucrose concentrations above 7.5% (w/v) reduced the cell growth (Narayan et al., 2002). Moreover, in cultures of *Gymnema sylvestre* with concentrations of 1% (w/v) and 3% (w/v) sucrose, fresh and dry weight (cellular density) were higher than for cultures with 5.7 or 9% (w/v) sucrose (Paek 2006). In this cases, higher sucrose concentrations resulted in an osmotic pressure affecting cell growth. The tested tamarillo non-embryogenic cellular lines, there wasn't a decline in cell growth, just a slow growth when compared to the cultures grown with 9% sucrose.

3.1.1. Cell suspension cultures growth with elicitors

In this section, elicitation of non-embryogenic cellular lines 1 and 2 is evaluated. As defined earlier, NEL 1 and NEL 2 were grown with two different biotic elicitors: casein hydrolyzed (CH) and yeast extract (YE). For both elicitors, two concentrations were tested: 0.5 g/L ([1]) and 1.5 g/L ([2]). Casein hydrolyzed was added to liquid medium in the beginning of growth and yeast extract during the major increase on cellular growth. Each non-embryogenic cellular line had a control sample (SM), a liquid culture grown without elicitor.

For each cellular line, a callus mass of 500 mg was added to 250 mL of liquid medium with 3% (w/v) of sucrose concentration. The first growth evaluation parameter for NEL 1 and NEL 2 cultures with CH and YE were the growth curves. Growth curves of NEL 1 cultures with elicitation are showed in figure 6.

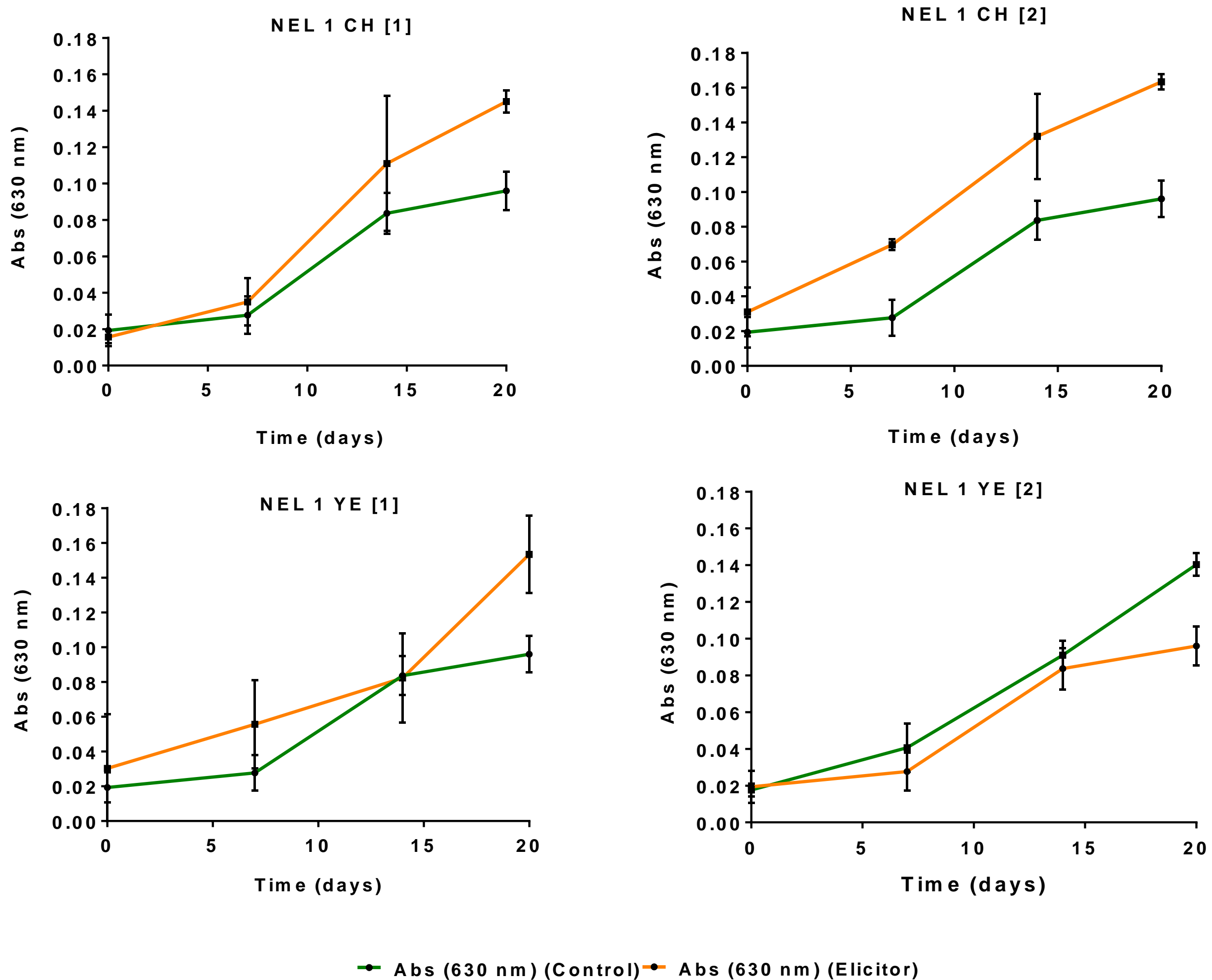

Figure 6. NEL 1 growth evaluation. Growth curves for SM, CH [1], CH [2], YE [1] and YE [2]. Data are represented as mean ± SD (n=3).

CH [1] growth curve, comparing with SM growth curve, started with a lower optical density media value, getting higher at day 7, and increasing until day 14. Between day 14 and day 20, CH [1] growth curve presented a lower slope, indicate a reduction on cellular growth. In this case, NEL 1 cultured with CH [1], for 20 days, demonstrated higher cellular

densities than NEL 1 cultured in control conditions (SM). In this case, the highest cellular growth was registered between day 7 and day 14. CH [2] cultures had higher optical density values in the beginning of growth than the control (SM), which was reflected in the next days of growth. Here, the differences between cellular densities were major than in CH [1] cultures. Again, since day 14 until day 20, it is observed a reduction on cellular growth. However, NEL 1 cells had a better growth in contact with CH [2] than without this elicitor (SM). Again, the highest growth was observed since day 14 until day 20. YE [1] and YE [2] elicitors were added to the liquid medium at day 14. YE [1] cultures had higher cellular densities than the control cultures during the 20 days of growth, however cultures for the addition of YE [1] behaved as the control until day 14. Then, one possible explanation is that in the 500 mg of callus used to the YE [1] cultures, the number of cells were higher than in SM cultures. However, this hypothesis will be clarified further. Since day 14, YE [1] cultures had an increase in optical density, translating in an increase on cellular growth. Cultures for the addition of YE [2] demonstrate lower optical density values than SM until day 14. The number of cells need to be appreciate, as in the case of YE [1], for further conclusions. However, YE [2] cultures, since day 14 (day when YE [2] was added), had an inferior optical density value than the control growth curve, with an inferior slope than the one registered before, suggesting a decrease on cellular growth.

Hereupon, in general, casein hydrolyzed elicitor at the two concentrations showed a better influence on cellular growth than yeast extract elicitor, when compared to the control.

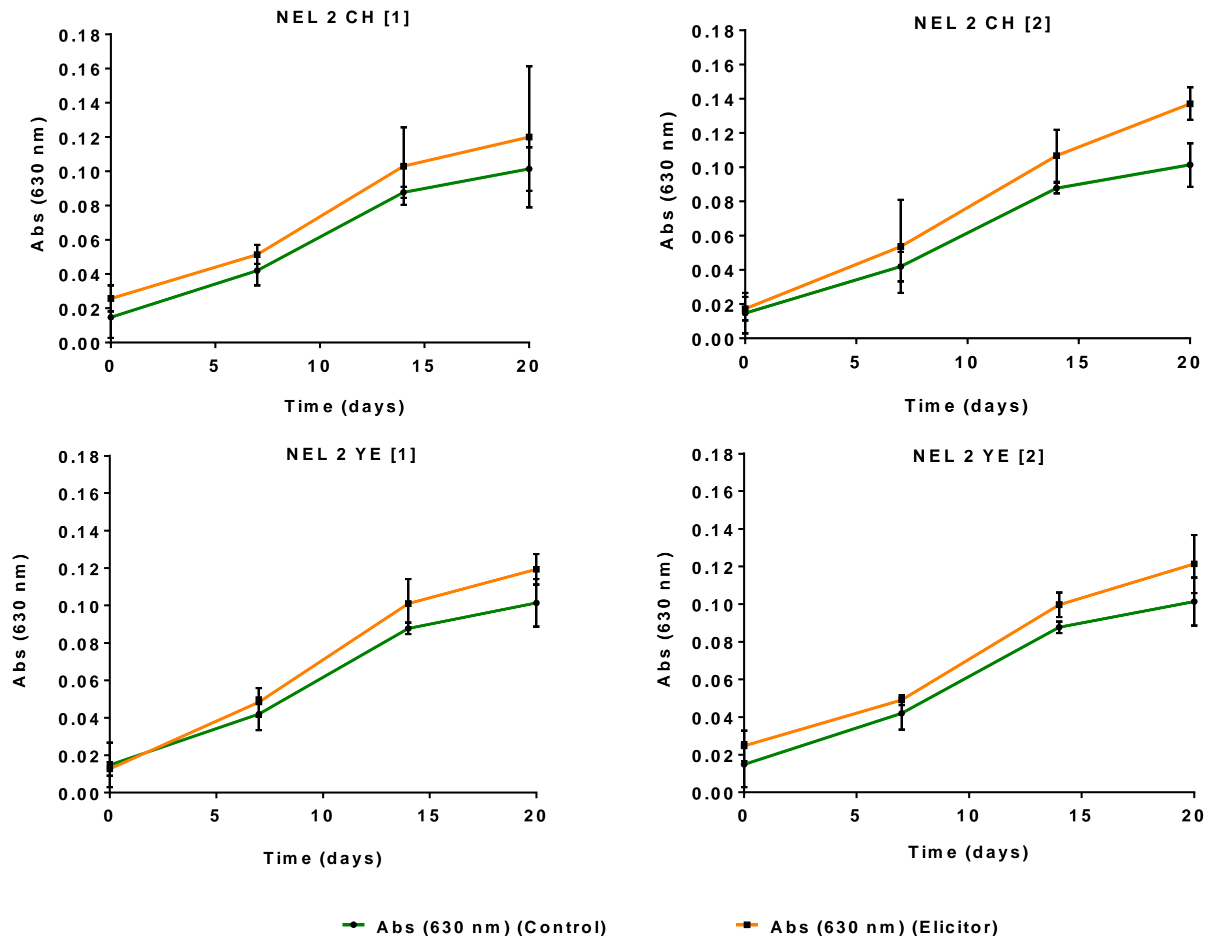

Figure 7. NEL 2 growth evaluation. Growth curves for SM, CH [1], CH [2], YE [1] and YE [2]. Data is represented as mean ± SD (n=3).

For NEL 2 cellular lines (Figure 7), the elicitor treatments showed high optical density values when compared to the control. Though, the elicitors' growth curves had a similar pattern to the control growth curves. CH [1] and CH [2] cultures had higher optical values than SM cultures, since the beginning of growth. However, between day 14 and day 20, there was a decrease on the curve slope, suggesting a decrease on cellular growth. For the two growth curves, the uppermost cellular growth was detected since day 7 until day 14. YE elicitor was added to the suspension cultures at day 11. It is possible to see that, even until day 11, the cultures for the addition of YE elicitor had higher cellular densities than SM cultures. However, the number of cells is needed to clarify this. So, since the day 11, YE [1] and YE [2] cultures continued with higher cellular densities than the control. In both curves,

since day 14, there was a decrease on cellular growth. For this non-embryogenic cellular line, all elicitors at the two concentrations showed a better behavior in terms of cellular density than the control.

As referred earlier, the next important evaluation on growth was the number of cells per mL of liquid culture. With this growth evaluation, the growth curves patterns could be clarified. So, figure 8 represents the number of cells for NEL 1 grown without (control) and with the two biotic elicitors.

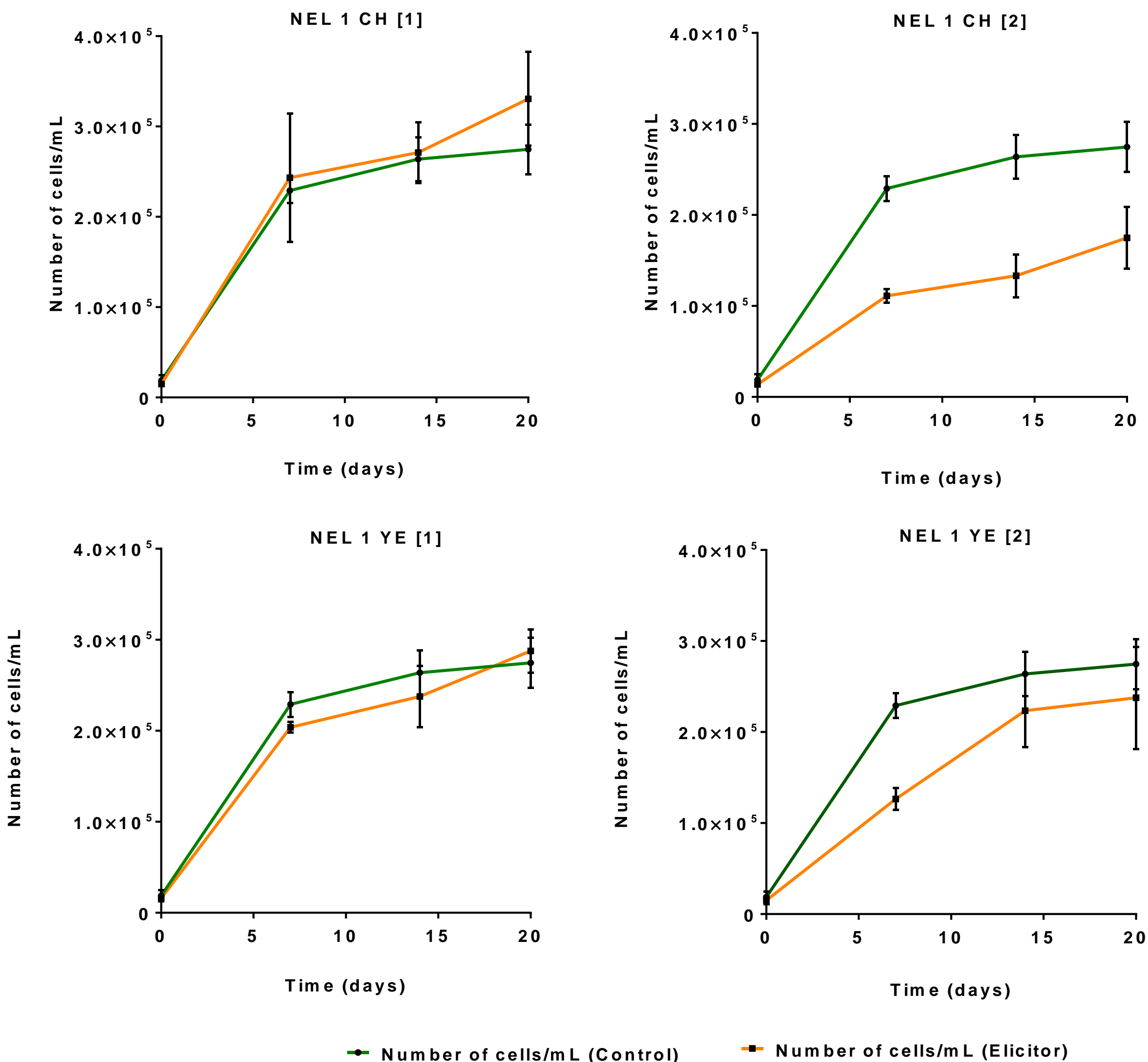

Figure 8. NEL 1 growth evaluation. Number of cells per mL of liquid medium for SM, CH [1], CH [2], YE [1] and YE [2]. Data is represented as mean ± SD (n=3).

Comparing to the control curve, only CH [1] presented higher number of cells. Though the growth curves of NEL 1 with elicitors (except YE [2]) had higher optical values than the control, the same didn't happen with the number of cells. CH [2], YE [1] and YE [2] cultures had lowest number of cells than SM cultures. In YE [2] cultures, the lowest number of cells since the beginning of growth justify the optical density values. In CH [2] cultures, the number of cells is slower than the SM cultures' number of cells. However, the cellular densities are higher than SM. So, the cellular growth of these cultures may be related to a phenomenon called aggregation. Aggregation can occur depending on the size of vegetal cells (the larger the cells the greater the tendency for the aggregation) or because of the presence of larger vacuoles or simply because the cells respond with aggregation to a strange stimulus present in the environment (elicitors) (Chattopadhyay et al. 2002). When aggregation occurs there is a difficult on counting the cells because of the cells' overlap in the counting chamber. Therefore, this lowest number of cells in CH [2] cultures can be related to an underestimation during the counting. In the case of YE [1] cultures, the number of cells increased more since day 14 until day 20, being higher than SM number of cells at day 20. So, in this circumstance, the number of cells had the highest increase three days after the addition of YE [1], which is in concordance with the cellular density of these cultures at this stage. Moreover, the SM cultures had higher number of cells since the beginning, but the cellular densities were higher in cultures for the addition of YE [1]. So, once again, there may have been an underestimation during the counting, because of the aggregation. In Figure 9 it is present the NEL 1 cells for SM, CH [1], CH [2], YE [1] and YE [2] cultures at day 20.

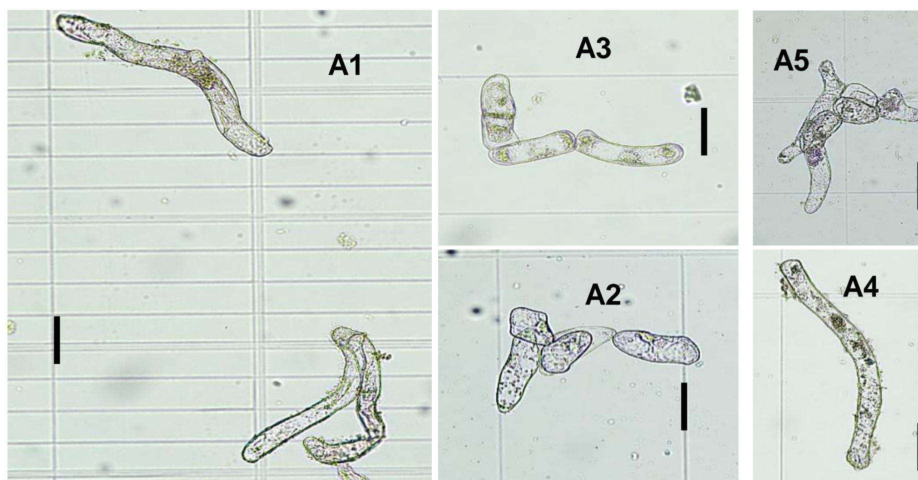

Figure 9. NEL 1 cells at day 20 (x100). A1 - SM; A2 – CH [1]; A3 – CH [2]; A4 – YE [1]; A5 - YE [2]. Bars correspond to 50 μm.

The same evaluation was made for NEL 2 (Figure 10).

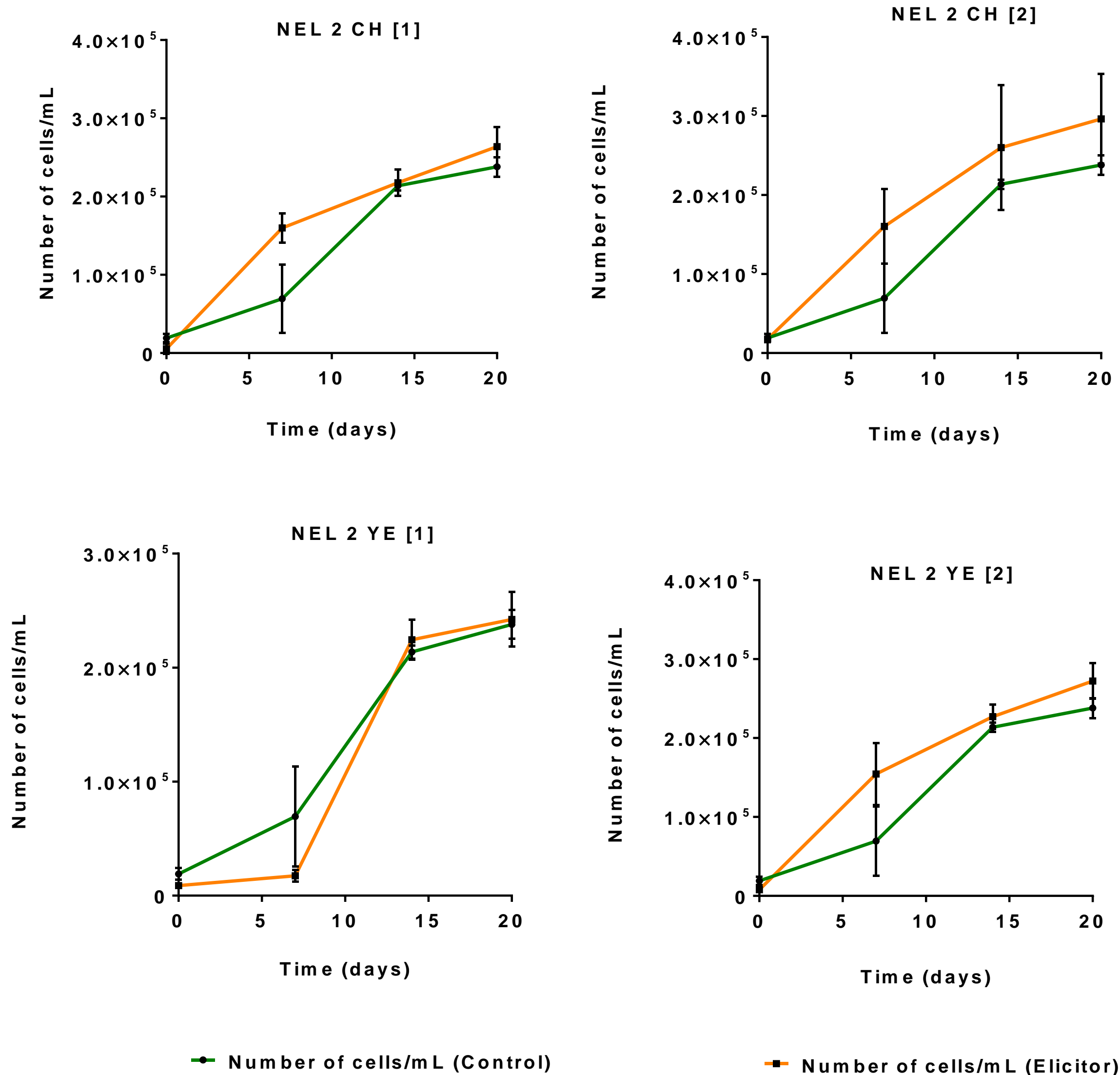

Figure 10. NEL 2 growth evaluation. Number of cells per mL of liquid medium. For SM, CH [1], CH [2], YE [1] and YE [2]. Data is represented as mean ± SD (n=3).

In CH [1], CH [2] and YE [2] cultures, the number of cells were superior to SM cultures. This justify the cellular densities which were higher in these cultures. In YE [1] cultures, the number of cells were highest since day 7 until day 14 and since day 14 until day 20, the number of cells were superior than SM number of cells. In this case, YE [1] was

added to the liquid medium at day 11, which can be related to the final increase in the number of cells. However, the SM cultures had higher number of cells since the beginning and YE [1] cultures had superior cellular densities since the beginning. So, once again, there may have been an underestimation during the counting of YE [1] cells, because of the aggregation. In Figure 11 are some examples of cells at day 20 of NEL 2.

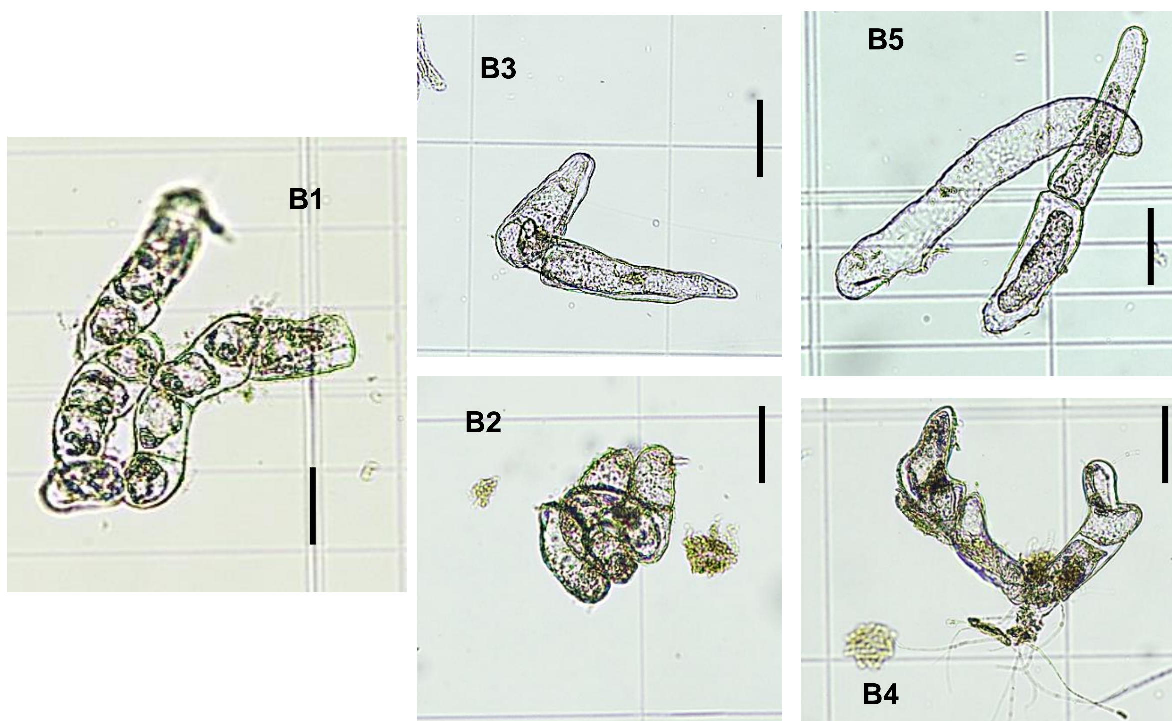

Figure 11. NEL 2 cells at day 20 (x100). B1 – SM; B2 – CH [1]; B3 – CH [2]; B4 – YE [1]; B5 – YE [2]. Bars correspond to 50 μm.

These results, growth curves and number of cells, demonstrated that casein hydrolyzed and yeast extract added to the culture medium influence the cell growth, having a better effect than the liquid medium without elicitation. In NEL 1 case, casein hydrolyzed in concentrations of 0.5 g/L and 1.5 g/L increased the cellular density since the begging of cultures growth. Yeast extract only was added to the cultures in day 14 and since this day,

only yeast extract in a concentration of 0.5 g/L increased the cellular growth. To understand if this improvement on cell growth was derived from the addition of the elicitors, the number of cells per mL of liquid culture was performed. This evaluation reveled those only cultures with CH [1] and YE [1] had higher number of cells than the control cultures. Accordingly to this, the improvement in cell growth in cultures with CH [1] and YE [1] could be related to the influence of these elicitors in these concentrations.

Now, in NEL 2 cultures, the two elicitors in the two concentrations improved the cellular density. Cultures with yeast extract and casein hydrolyzed had higher growth rates since the beginning of culture. However, yeast extract was only had to the culture at day 11. To correlate these with the influence of the elicitors, the number of cells was registered too. What was observed was that casein hydrolyzed cultures (0.5 g/L and 1.5 g/L) had higher number of cells than the control. In the case of yeast extract cultures, cultures with 0.5 g/L had more cells only since the addition of yeast extract and cultures with 1.5 g/L had more cells since the beginning of cultures growth. So, the improvement in cultures' cell growth with casein hydrolyzed and with yeast extract 0.5 g/L could be related to the addition of these elicitors and the higher rates in cellular density since the beginning on cultures with yeast extract 1.5 g/L could be related to the aggregation phenomenon.

By the observation of the cells, the same cellular features were possible observe to both cell types in all the conditions tested and the control (Figures 9, 10 and 11), namely the elongated and irregular shape of the cells, their large vacuoles and the tendency to form cell aggregates.

Several studies have been built based on the use of elicitors to improve the cellular density on cell suspension cultures. One of these studies made on *Panax vietnamensis* cultures treated with yeast extract and casein hydrolyzed revealed that 1.0 g/L of yeast extract and 2.0 g/L of casein hydrolyzed produced more cellular density. Moreover, in these cultures casein hydrolyzed had a better effect that yeast extract (Trong et al. 2017).

In our case, and in concordance with the results obtained with *Panax vietnamensis*, casein hydrolyzed had a better behavior on the cellular growth than yeast extract in both cellular lines.

3.2. Protein analysis

3.2.1. Intracellular protein

The next step was to define an effective protein extraction method for NEL 1 and NEL 2 cells. Four protein extraction processes were performed: mechanic treatment with a plastic piston, mechanic treatment with liquid nitrogen, mechanic treatment with glass beads and enzymatic treatment with pectinase enzyme. The first method tested was the mechanic treatment with a plastic piston. To test this method, NEL 1 and NEL 2 cells grown at the two sucrose concentrations (M9 and M3) were used. Apart from this, it was important to evaluate the protein content in both sucrose concentrations. So, intracellular protein concentration and callus mass were evaluated at day 14 (final day of cellular growth) where liquid cultures were filtered in order to separate the cells from the liquid medium. Cells collected (callus) were weighted and then macerated. In Table I it is represented the protein concentration (µg/mL) and protein quantity (µg) in NEL 1 and NEL 2 cells and the final callus mass at the two sucrose circumstances. The protein content was evaluated using Bio-Rad Protein Assay protocol, as described in material and methods chapter.

Table I. Intracellular protein concentration (µg/mL), protein quantity (µg) and final callus mass (mg) of NEL 1 and NEL 2 in M9 and M3. Results are presented as mean ± SD (n=3).

	Protein concentration (µg/mL) ± SD	**Protein quantity (µg) ± SD**	**Final callus mass (mg) ± SD**
NEL 1 M9	225.62 ± 107.25	360.99 ± 171.60	594.30 ± 2.55
NEL 1 M3	50.51 ± 17.66	80.82 ± 28.25	593.77 ± 3.85
NEL 2 M9	114.89 ± 46.70	183.82 ± 74.72	594.33 ± 8.63
NEL 2 M3	66.24 ± 10.36	105.99 ± 16.57	594.57 ± 9.73

Cells protein concentration (µg/mL) was higher in cell lines grown in M9 and the difference of protein obtained between sucrose concentrations for both lines was higher in NEL 1, which was reflected in protein quantity. In terms of callus final mass (mg), the values are similar.

With these results it was important to relate the protein quantity per mg of callus (µg/mg) both non-embryogenic cellular lines at M9 and M3 (Figure 12).

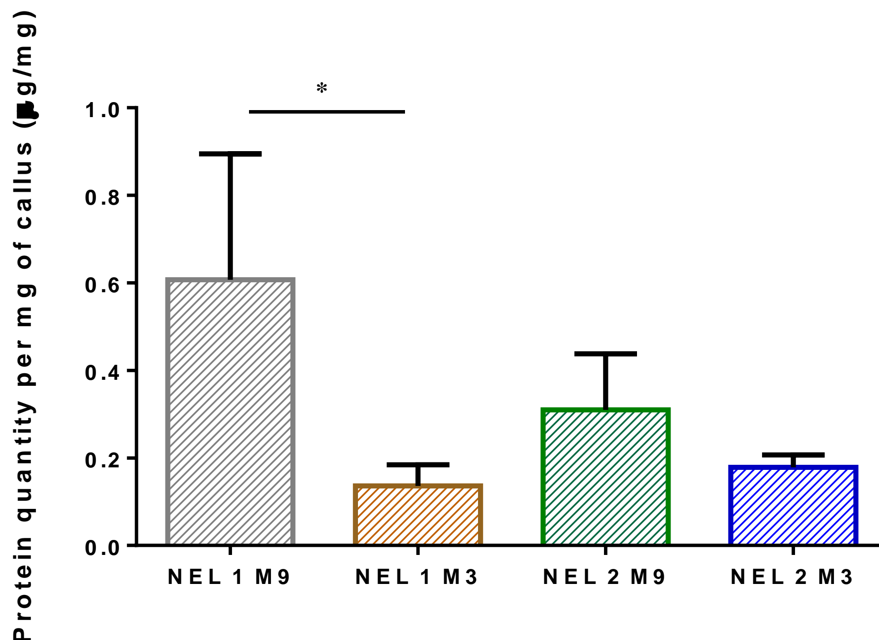

Figure 12. Protein quantity per mg of callus (µg/mg) for NEL 1 and NEL 2: M9 - 9% (w/v) sucrose; M3 - 3% (w/v) sucrose. Data presented as mean ± SD (n=3). Statistical analyze with unpaired t test ($p<0.05$): **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

For NEL 1 there is a significant statistical difference between the two sucrose concentrations used, preferring a concentration of 9% (w/v) sucrose instead of 3% (w/v). For NEL 2 there are no significant statistical differences. Moreover, the protein quantity per mg of callus is higher in NEL 1 than in NEL 2. Another important note present in Table I and in figure 12 is that NEL 2 better adapted to a change in sucrose concentration. This may related to the *in vitro* establishment, because NEL 2 was established long before NEL 1 and was maintained in culture during several time.

The other three protein extraction methods (liquid nitrogen, pectinase and glass beads) were performed next. NEL 2 was not used in this assay once there wasn't enough callus biomass to perform these assays. Replicas of NEL 1 cells grown with 3 % (w/v) sucrose were used. All these protein extraction procedures were made only at cellular growth final day. Protein content was estimated using Bio-Rad Protein Assay protocol. In

Table II it is indicated protein quantity (µg) and protein concentration (µg/mL) as well as final callus mass for these protein extraction methods.

Table II. Intracellular protein concentration (µg/mL), protein quantity (µg) and final callus mass (mg) of samples treated with liquid nitrogen, pectinase and glass beads. Results are presented as mean ± SD (n=3).

	Protein concentration (µg/mL) ± SD	**Protein Quantity (µg) ± SD**	**Final callus mass (mg) ± SD**
Mechanic Treatment (Liquid Nitrogen)	582.11 ± 197.91	363.82 ± 123.70	620 ± 17.32
Enzymatic Treatment (Pectinase)	47.60 ± 11.73	29.75 ± 7.33	606.67 ± 5.77
Mechanic Treatment (Glass Beads)	19.57 ± 4.94	12.23 ± 3.09	650 ± 20

Cells macerated with liquid nitrogen, had the highest protein concentration value, reflecting a highest protein content value. Enzymatic treatment with pectinase released a lower protein quantity (lower protein concentration) and mechanic treatment with glass beads was the treatment less effective. In terms of final callus mass, the values among replicas were similar, being slightly superior in samples treated with liquid nitrogen and with glass beads.

In Figure 13, protein quantity per mg of callus (µg/mg) is distinguished for both protein extraction methods.

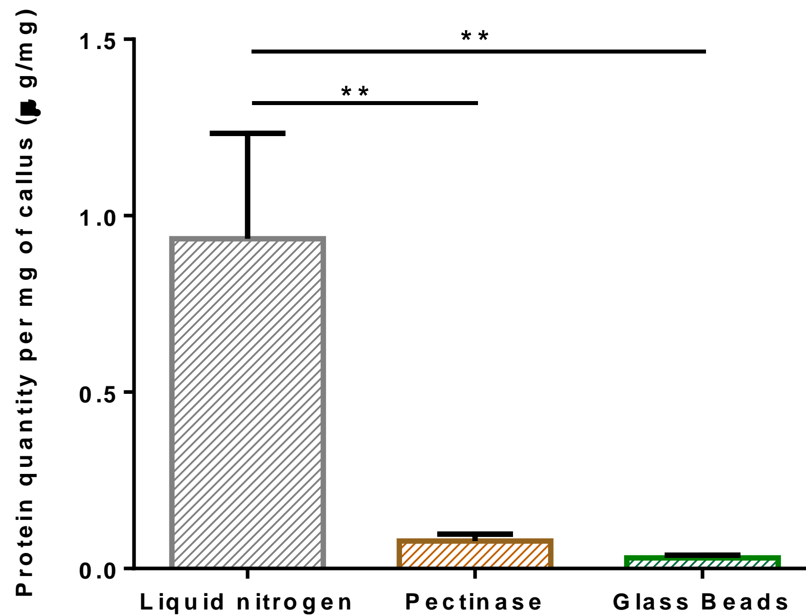

Figure 13. Protein quantity per mg of callus (µg/mg) for samples treated with liquid nitrogen, pectinase and glass beads. Data presented as mean ± SD (n=3). Statistical analyze with Tukey test (p<0.05): **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05.

Mechanic treatment with liquid nitrogen had the major protein quantity per mg of callus, showing significant statistical differences when compared to the other methods, which is in concordance with previous results.

In this topic the objective was to find an effective cells' protein extraction and which sucrose concentration was better to grow NEL 1 and NEL 2 cells. As indicated in Table I, M9 medium resulted in a higher intracellular protein content that M3 medium. This result is in concordance with the growth curves evaluated earlier. Apart from these, NEL 2 better adapted to a change in sucrose concentration. This may related to the *in vitro* establishment. Now, accordingly to the extraction method, it is denoted that mechanic treatment with liquid nitrogen is the most suitable method to obtain the protein content from cells instead of the use of a plastic piston, glass beads or pectinase. Although non-embryogenic cellular line 2 was not used, the method protocol can be applied to this cellular line.

The next step was the calculation of the protein content in NEL 1 and NEL 2 with elicitation. So, before the maceration of cells with liquid nitrogen, they were weighted to appraise the final callus mass. After the maceration, sodium phosphate buffer 0.05 M pH 7 was added to the cells to further evaluation with Bio-Rad Protein Assay protocol. In Table III it is indicated protein quantity (µg) and protein concentration (µg/mL) as well as final callus mass of NEL 1 and NEL 2 in control and elicitation condition.

Table III. Intracellular protein concentration (µg/mL), protein quantity (µg) and final callus mass (mg) of NEL 1 and NEL 2 SM, CH [1], CH [2], YE [1] and YE [2]. Results are presented as mean ± SD (n=3)

	Protein concentration (µg/mL) ± SD	Protein Quantity (µg) ± SD	Final callus mass (mg) ± SD
SM NEL 1	15.53 ± 5.95	73.60 ± 33.25	615.67 ± 71.67
CH [1] NEL 1	62.11 ± 22.01	280.75 ± 96.93	666.67 ± 90.19
CH [2] NEL 1	42.15 ± 16.78	201.28 ± 65.10	579.67 ± 43.94
YE [1] NEL 1	10.55 ± 14.51	57.46 ± 80.25	552 ± 33.05
YE [2] NEL 1	15.35 ± 17.00	82.69 ± 92.28	617.33 ± 34.08

	Protein concentration (µg/mL) ± SD	Protein Quantity (µg) ± SD	Final callus mass (mg) ± SD
SM NEL 2	16.16 ± 2.53	84.45 ± 2.39	569 ± 14.42
CH [1] NEL 2	8.30 ± 6.32	48.09 ± 45.99	586 ± 26.67
CH [2] NEL 2	49.05 ± 10.63	244.86 ± 70.99	640 ± 55.68
YE [1] NEL 2	2.49 ± 3.37	16.64 ± 24.18	568 ± 20.30
YE [2] NEL 2	0.36 ± 0.22	2.47 ± 2.30	584.33 ± 16.92

Starting with NEL 1, CH [1], CH [2] and YE [2] had more protein quantity (µg) than SM NEL 1 (control). Only YE [1] had a lower value than SM. Comparing the two concentrations for each elicitor, CH [1] had superior protein content than CH [2] and YE [2]

had a major protein quantity value than YE [1]. In relation to NEL 2, only CH [2] had a higher value than the control. Again, relating the two concentrations for each biotic elicitor, CH [2] had more protein than CH [1] and YE [1] presented more protein than YE [2]. It is notable that the two non-embryogenic lines had different behaviors when in elicitation. Now in terms of final callus mass for NEL 1, sample CH [1] showed a higher mass than the control sample, which can be related to the higher value of protein content. For YE [1] and YE [2] the final callus mass is in concordance with the protein quantity. For NEL 2, only sample YE [1] had a lower value than the control sample and comparing these results to the number of cells described earlier, this sample was the one with lowest values when compared to the control. Moreover, CH [2] had a higher callus mass value than the control, which is in concordance with the protein quantity for this elicitor at this concentration. It is important to refer that for both lines protein content of YE [1] and YE [2] had standard values higher than mean, once the protein content among replicas were not homogeneous. In conclusion, for NEL 1 CH [1] produced more callus mass and more protein than the control.

Said this, an important evaluation was needed to clarify previous results: protein quantity per mg of callus (µg/mg). Protein quantity in relation callus mass (µg/mg) for NEL 1 and NEL 2 is distinguished in Figure 14.

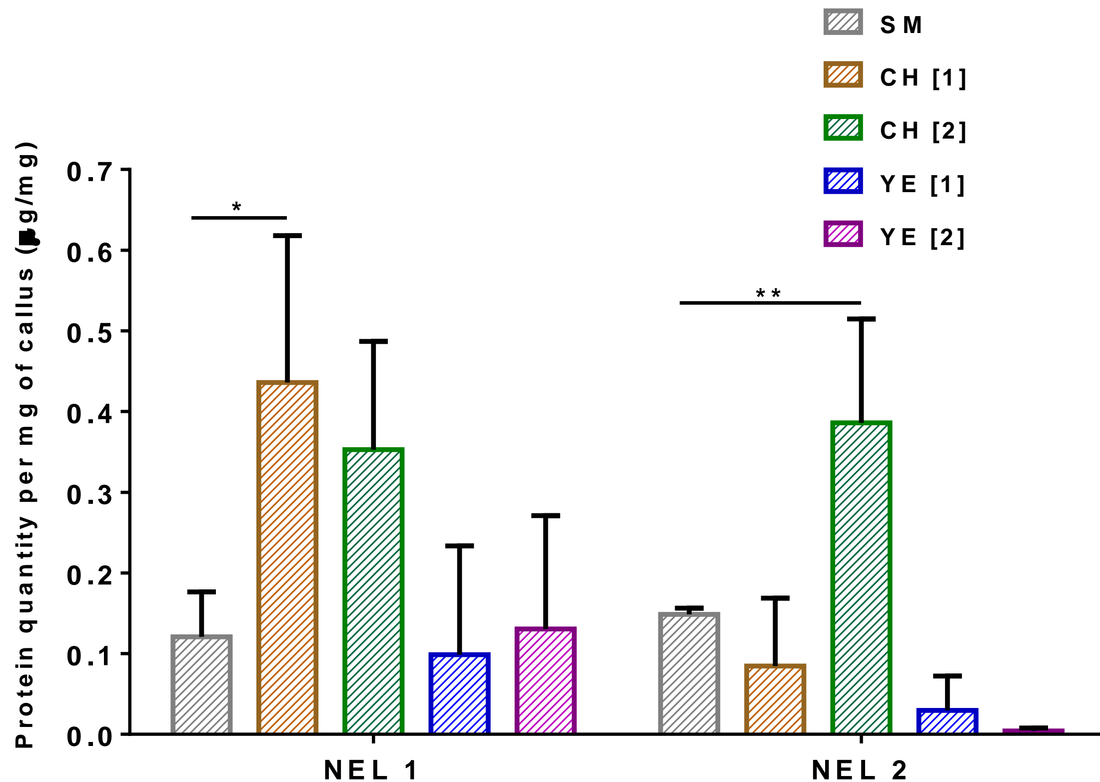

Figure 14. Protein quantity per mg of callus (µg/mg) for NEL 1 and NEL 2 SM, CH [1], CH [2], YE [1] and YE [2]. Data presented as mean ± SD (n=3). Data analyzed with Dunnett's multiple comparisons test (p<0.05): **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05.

Elicitors' protein quantity per mg of callus was compared to the control by Dunnett's multiple comparisons test (p<0.05). This graph reflects the earlier observations where CH [1] elicitor produce more protein in NEL 1 and CH [2] elicitor produced more protein in NEL 2. The differences against the control sample had more significance in NEL 2.

3.2.2. Extracellular protein

As referred earlier, NEL 1 and NEL 2 cultures were filtered in order to separate the liquid medium from the cells. The liquid medium was dialysate and lyophilized. After these processes, the protein content was evaluated with Bio-Rad Protein Assay protocol. NEL 1 and NEL 2 M9 and M3 liquid mediums didn't revealed the presence of proteins. Moreover, the analysis with Bio-Rad Protein Assay protocol of the liquid mediums of NEL 1 and NEL 2

cultures in contact with CH [1], CH [2], YE [1] and YE [2] didn't showed any protein manifestation.

3.2.3. Protein profiles

3.2.3.1. SDS-PAGE Profiles

In order to analyze the intracellular protein content in NEL 1 and NEL 2 cultures with elicitation, 12.5 % SDS-PAGE gels were performed. In addition, a protein concentration protocol with chloroform and methanol was achieved. In figure 15, 12.5 % SDS-PAGE gels of NEL 1 and NEL 2 are represented.

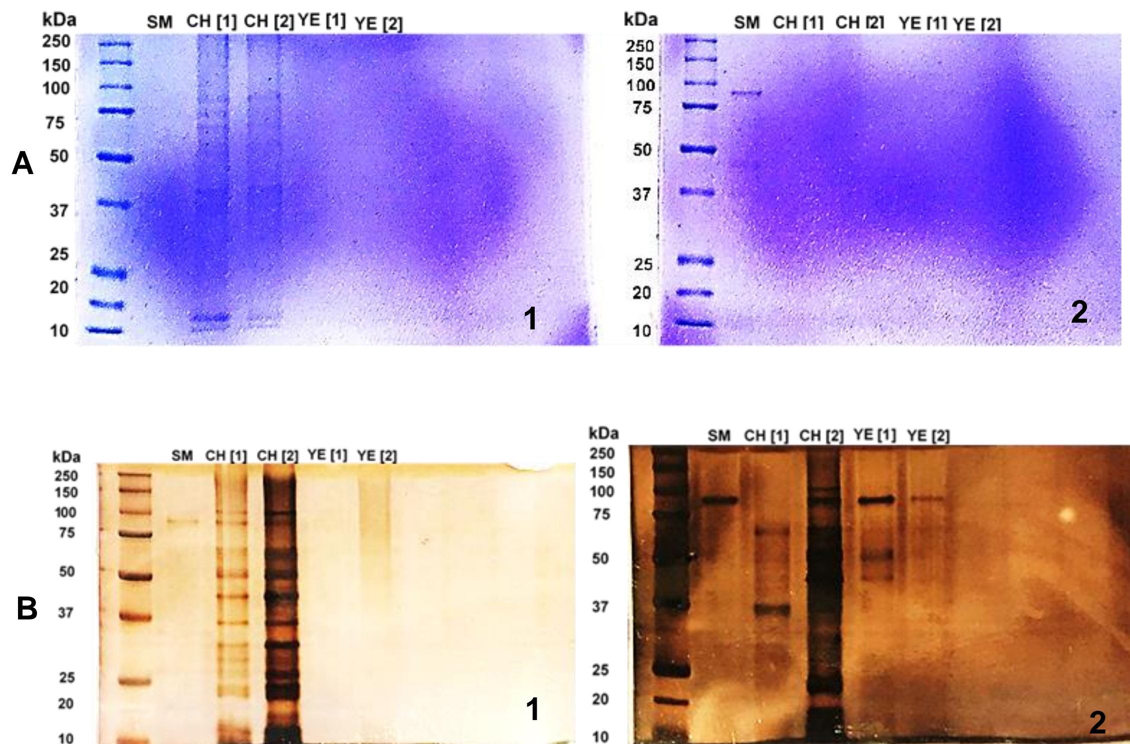

Figure 15. NEL 1 and NEL 2 12.5 % SDS-PAGE gels (SM, CH [1], CH [2], YE [1] and YE [2]). (A) 12.5 % SDS-PAGE gels stained with Coomassie Blue, (1) NEL 1 samples and (2) NEL 2 samples; (B) 12.5 % SDS-PAGE gels stained with silver nitrate, (1) NEL 1 samples and (2) NEL 2 samples.

Gels stained with Coomassie Blue (Figure 15A) showed more protein quantity for NEL 1 CH [1] and CH [2] samples and for NEL 2 SM and CH [2] samples. In SM NEL 2 sample, three bands are visible: one at 37 kDa, another between 50 and 75 kDa and another one between 75 and 100 kDa. In CH [1] and CH [2] a lot of bands appeared in gel. Now, in figure 15B it is showed the gels in Figure 15A, but stained with silver nitrate. More bands appeared with silver nitrate treatment. These gels are in concordance with the previous ones, where CH [1] and CH [2] of NEL 1 had highest protein quantity. Moreover, in NEL 1 SM sample, a band between 75 and 100 kDa appeared too, which may be the same protein that appeared in NEL 2 SM sample. NEL 1 CH [1] and CH [2] samples had the presence of the same bands: one at 100 kDa, another between 75 and 100 kDa, other one between 50 and 75 kDa, another at 50 kDa, other between 37 and 50 kDa, another at 37 kDa, three bands between 37 and 25 kDa, two bands between 25 and 20 kDa, two between 20 and 10 kDa and another one at 10 kDa.

Moreover, NEL 2 gel stained with silver nitrate revealed that the other samples (CH [1], YE [1] and YE [2]) had proteins too. In NEL 2 CH [1] there are three bands that stands out: one at 37 kDa, another at 50 kDa and other one between 50 and 75 kDa. In NEL 2 CH [2] samples a lot of bands appeared, but six of the bands appeared more clearly: one at 100 kDa, another between 75 and 100 kDa, other one between 50 and 75 kDa, other between 37 and 50 kDa, another at 37 kDa and another one between 25 and 20 kDa. YE [1] sample presented three bands: one between 75 and 100 kDa, other at 50 kDa and another one between 37 and 50 kDa. In the case of YE [2] sample only a band between 75 and 100 kDa appeared.

In conclusion, it is possible to see that inside the same non-embryogenic cellular line, samples have similar bands, that possible could be the same proteins. Moreover, the protein profiles are different between NEL 1 and NEL 2, but there is a clear similarity in SM samples (one band between 75 and 100 kDa). Apart from this, the samples with more protein quantity and with more bands are in concordance with the intracellular protein analysis, described earlier.

As referred earlier, liquid mediums didn't revealed the presence of proteins with the Bio-Rad Protein Assay protocol. However, to confirm this result, SDS-PAGE gels for liquid medium were made too (Figure 16). Moreover, in these SDS-PAGE gels were included samples from liquid mediums with elicitors.

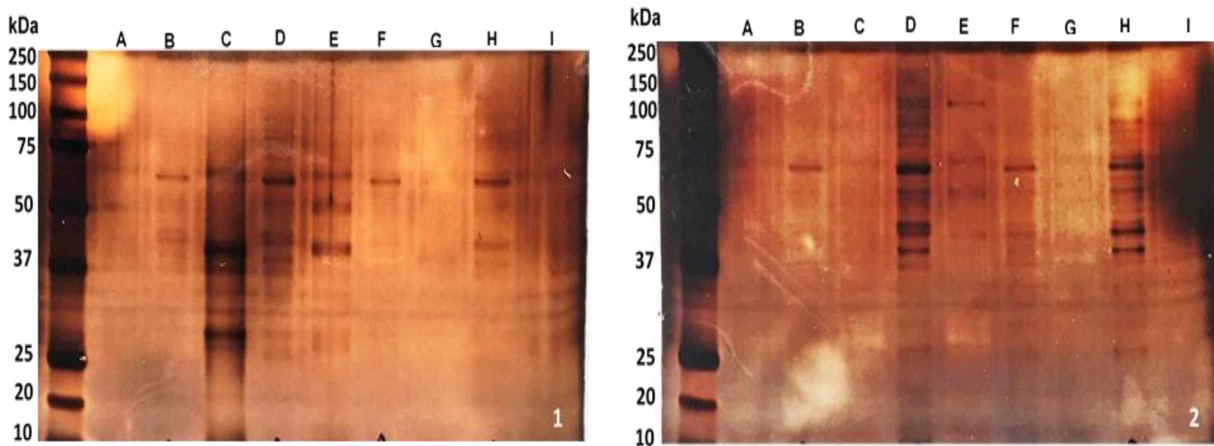

Figure 16. 12.5 % SDS-PAGE gels of NEL 1 and NEL 2 liquid mediums stained with silver nitrate**.** (1) NEL 1 and (2) NEL 2. A – SM; B – 0.5 g/L of casein hydrolyzed; C – CH [1]; D – 1.5 g/L of casein hydrolyzed; E – CH [2]; F – 0.5 g/L of yeast extract; G – YE [1]; H – 1.5 g/L of yeast extract; I – YE [2].

Samples of liquid mediums with elicitors were applied to the gels with control samples. That is, yeast extract and casein hydrolyzed elicitors are two substances that are constituted by proteins and amino acids. So, to despite the presence of these compounds from elicitors in liquid mediums, control samples were performed with casein and yeast extract at the two concentrations for the two non-embryogenic cellular lines and applied to the gels. Letters B, D, F and H correspond to these control samples and letters A, C, E, G and I correspond to the liquid mediums samples with elicitation.

In SM samples, that are the samples of NEL 1 and NEL 2 without elicitation (liquid medium with a concentration of 3 % (w/v) sucrose) no bands appeared in gels, suggesting that cells in SM cultures didn't secret any proteins to the medium and/or that these cells didn't suffer lysis that could release the intracellular proteins to the liquid medium. Now, comparing the control samples to the liquid mediums samples of NEL 1 and NEL 2, the bands that appeared in liquid mediums samples are identical to those observed in control

samples. This suggest that the proteins present in liquid mediums are from yeast extract and casein hydrolyzed. Moreover, this means that the cells, even in the presence of these two elicitors, didn't secret any proteins to the liquid mediums.

3.2.3.2. Zymography

Now, as the presence of proteins in liquid mediums of NEL 1 and NEL 2 was deleted, another important protein evaluation was made to the intracellular samples: a zymography. This technique performed include a gel with 12.5% polyacrylamide and gelatin, an enzymatic substrate for proteolytic enzymes. So, this method was achieved in order to verify the presence of proteolytic enzymes in intracellular samples. Figure 17 represents the zymographys of NEL 1 and NEL 2 samples.

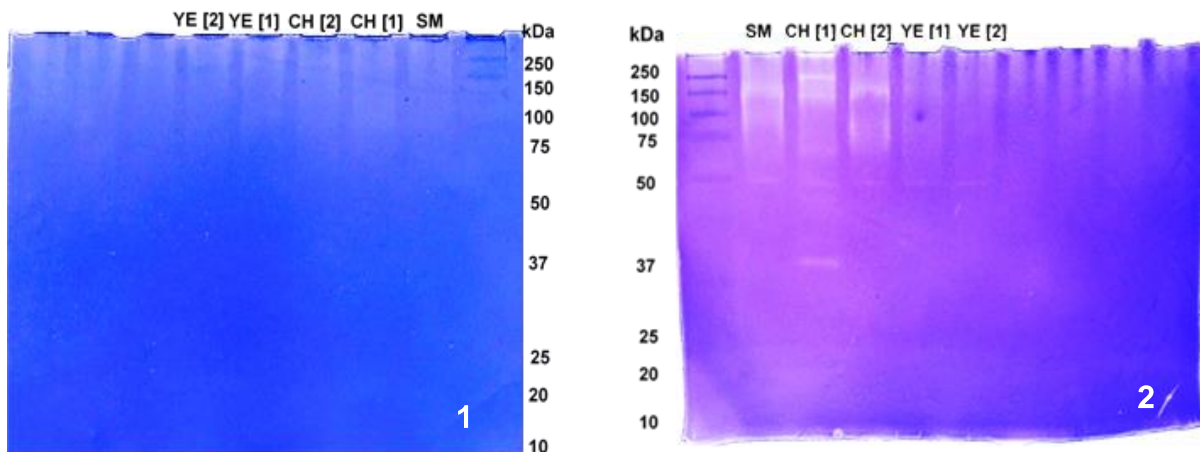

Figure 17. Zymographys of NEL 1 (1) and NEL 2 (2) intracellular samples (SM, CH [1], CH [2], YE [1] and YE [2]).

Zymography of NEL 1 didn't reveled any bands (1) for any sample. However, NEL 2 zymography reveled bands for all samples. In SM sample, a band between 75 and 100 kDa appeared, probably the same protein that appeared in SDS-PAGE gel demonstrated earlier. But, despite of what happened in SDS-PAGE gel of NEL 2, here two more bands appeared in SM: one at 150 kDa and another band at 50 kDa.

For CH [1] sample five bands were observed: one at 250 kDa, another at 150 kDa, one between 50 and 75 kDa, one at 50 kDa and another one at 37 kDa. Bands between 50 and 75 kDa, at 50 kDa and at 37 kDa probably correspond to the proteins identified previously for this sample in SDS-PAGE gel. CH [2] sample presented three bands: one at 150 kDa, other one between 100 and 75 kDa and finally one at 50 kDa. Accordingly to the previous SDS-PAGE gel (were a lot of bands appeared for this sample) just the band at 150 kDa doesn't appear. YE [1] and YE [2] samples had the same band that is located at the molecular weight of 50 kDa. Once again, this band appear in SDS-PAGE gel for YE [1] sample, but not for YE [2] sample. These bands that appeared in NEL 2 zymography gel, but does not appeared in SDS-PAGE gel can be related to the low amount of that proteins in the intracellular medium. Though, SM, CH [1] and CH [2] samples are the samples in which the presence of proteolytic enzymes were more accented. Moreover, NEL 1 samples didn't reveled any bands, which suggest that this cellular line had low protein quantity and/or low protease activity.

3.2.3.3. Proteolytic profiles

The next goal was to evaluate the protein catalytic activity on NEL 1 and NEL 2 cells. First, cells of NEL 1 and NEL 2 grown with M9 and M3 were evaluated. For that, a enzymatic assay with a set of fluorogenic substrates were essayed. The first group of fluorogenic substrates tested was centralized in the detection of proteases having a fluorogenic group in C-terminal: amino methylcoumarine (AMC). Four substrates were tested: Phe-AMC, Arg-AMC, Met-AMC and Lys-AMC, in the conditions described earlier. These four substrates were used and not others because previous works (Alves et al. 2017) demonstrated that tamarillo callus in suspension cultures had highest proteases activity with these substrates. Activity results are expressed in specific activity. All enzymatic substrates showed activity on all extracts (Figure 18).

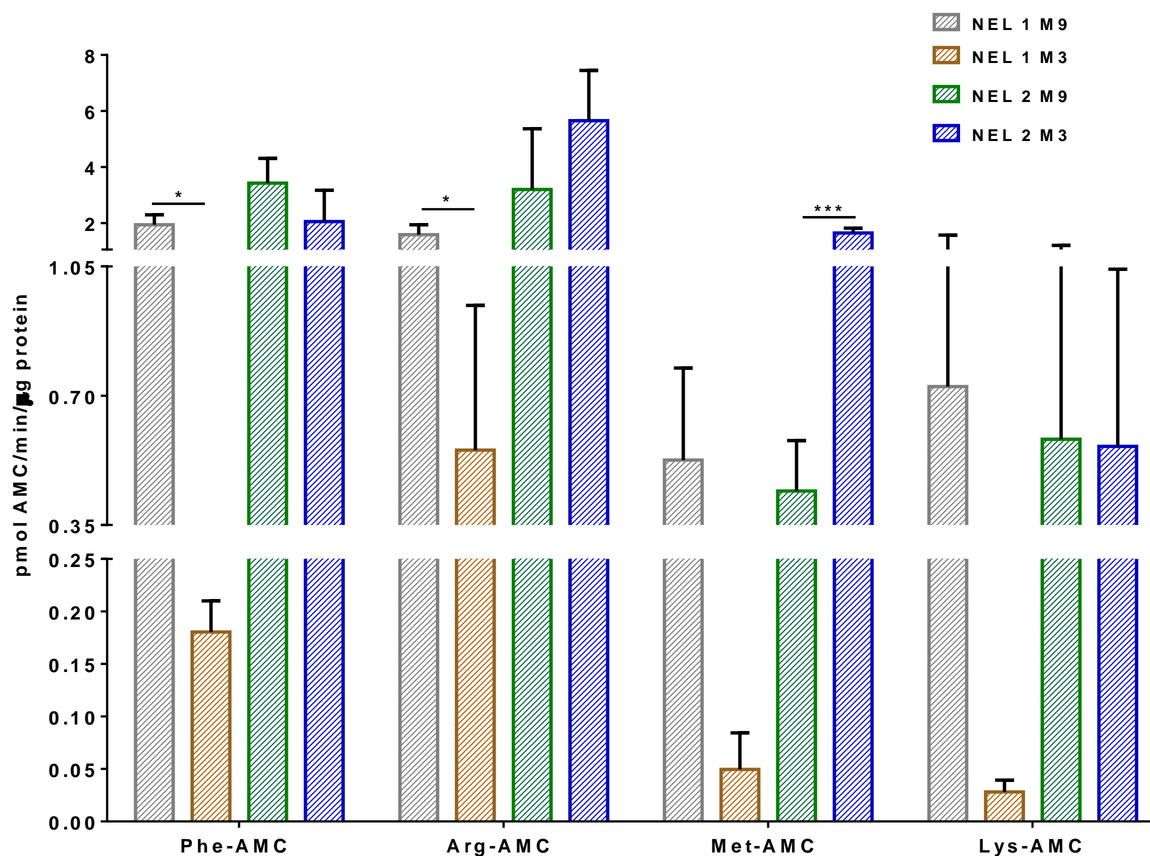

Figure 18. Specific Phe-AMC, Arg-AMC, Met-AMC and Lys-AMC activity of NEL 1 and NEL 2: M9 - 9% (w/v) sucrose; M3 - 3% (w/v) sucrose. Data presented as mean ± SD (n=3). Statistical analyze with unpaired t test (p<0.05): **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05

Phe-AMC and Arg-AMC enzymatic substrates were the two substrates tested with higher specific activity values. Within Phe-AMC, NEL 1 presented a statistical difference between M9 and M3, high in M9. NEL 2 do not showed statistical difference, but the activity mean was high in M9 than in M3. However, with this enzymatic substrate, NEL 2 exhibited a higher activity when compared to NEL 1. With Arg-AMC, the activity pattern was similar to the one previously described. Again, in NEL 2 there were no differences, however here the activity pattern inverted, comparing to the one obtained with Phe-AMC. Once again, NEL 2 presented a higher activity than NEL 1. The activity with Met-AMC was lowest when compared to the previously substrates scrutinized and NEL 1 M9 showed an increase on

activity bigger than NEL 1 M3 (with no statistical significance) and in NEL 2, M3 presented a very significant difference to M9. Lys-AMC substrate does not showed statistical differences between M9 and M3 for both lines, a consequence of variance heterogeneous values. However, the activity patterns were similar to the ones previously described (more activity in NEL 1 M9 and in NEL 2 M9.

Comparing both non-embryogenic cellular lines at the two sucrose concentrations, the patterns of activities are quite similar. This suggests that the types of proteases present in cells are similar between cellular lines. The highest and lowest values of activity could be related to the amount of those proteins inside the cells. Proteins in NEL 1 M9 reveled more protease activity than proteins in NEL 1 M3. However, only with Phe-AMC and Arg-AMC there was statistical differences. Furthermore, proteins presented in NEL 2 cells showed more protease activity than proteins in NEL 1 cells. Moreover, the statistical differences found between M9 and M3 in NEL 2 were not significant (except in Met-AMC), indicating that this cellular line is capable to produce the identical amount of proteins, in this case of proteases, when there is a change in the sucrose concentration of the medium.

Another group of fluorogenic substrates was essayed for cells protein samples: MU-NAG, MU-C, MU-P and MU-G (Figure 19). Once again, these four substrates were used and not others because previous works (Alves et al. 2017) demonstrated that tamarillo callus in suspension cultures had highest activity with these substrates.

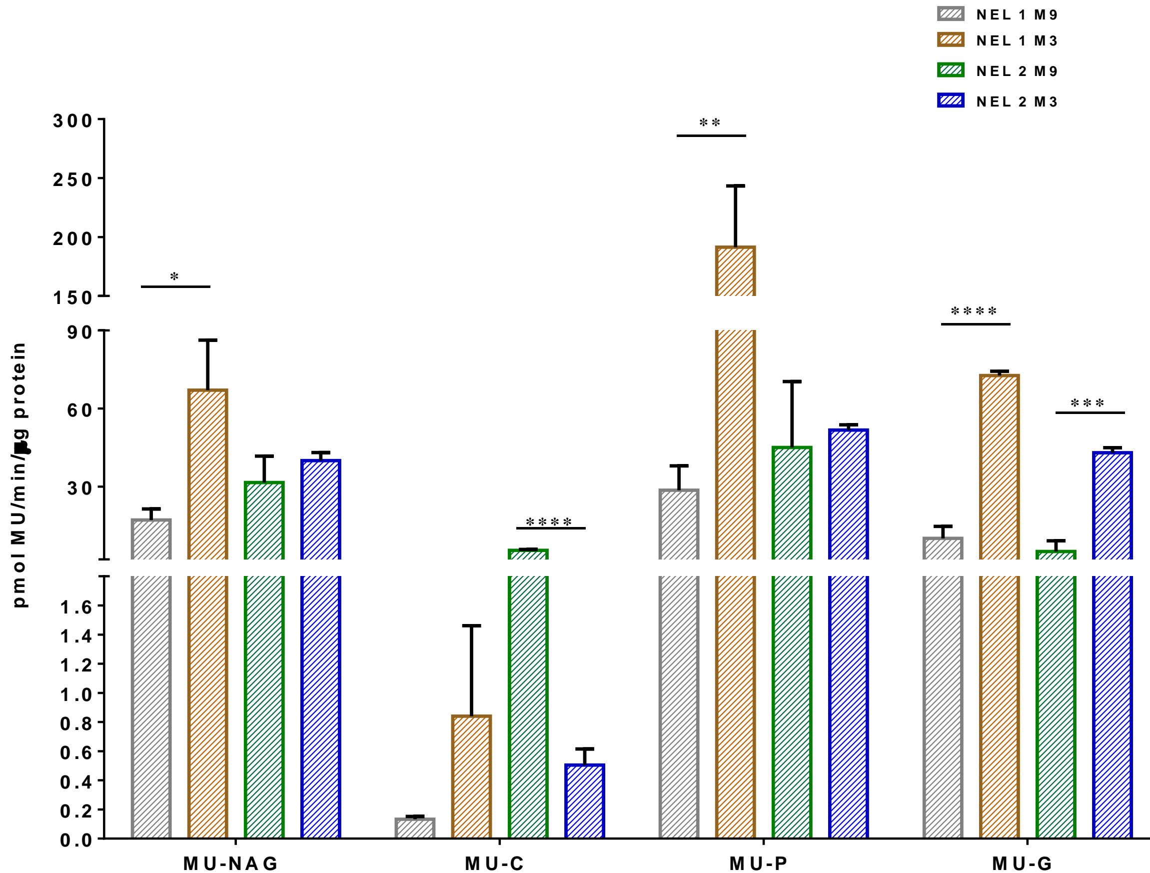

Figure 19. Specific MU-NAG, MU-C, MU-P and MU-G activity of NEL 1 and NEL 2: M9 - 9% (w/v) sucrose; M3 - 3% (w/v) sucrose. Data presented as mean ± SD (n=3). Statistical analyze with unpaired t test (p<0.05): **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05.

As described in material and methods chapter, MU-NAG, MU-C and MU-G are substrates to evaluate the presence of glycoside hydrolases and MU-P to alkaline phosphates. All substrates reveled activity on all samples. MU-NAG, MU-P and MU-G had the highest activity values.

Starting with MU-NAG, NEL 1 M3 showed an increase on specific activity bigger than NEL 1 M9. NEL 2 doesn't exhibited statistical difference between M9 and M3. Both non-embryogenic cellular lines had similar activity values, being higher in M3. MU-C activity values were the lowest and NEL 1 didn't reveled statistical difference between the two sucrose levels, a result of heterogeneous variances. NEL 2 M9 had a higher specific activity

than NEL M3, showing a significant statistical difference. The alkaline phosphatases (MU-P) activity presented a higher activity in NEL 1 M3 than in NEL 1 M9. In NEL 2, comparing M9 to M3, there wasn't statistical difference, once again due to the non-similar variances. MU-G was the only substrate that demonstrated statistical differences between M9 and M3 in both non-embryogenic cellular lines: NEL 1 M3 and NEL 2 M3 had greater specific activity values when compared to M9.

Again, comparing both non-embryogenic cellular lines at the two sucrose concentrations, the patterns of activities reveled some differences. In this case both cellular lines had more activity in M3 than in M9 (except with MU-C for NEL 2).

So, Phe-AMC and Arg-AMC were the two enzymatic substrates preferred by both cellular lines. So both cellular lines showed that the type of proteases present in them prefer cut enzymatic substrates with phenylalanine and arginine amino acids. With MU substrates, both cellular lines showed more activity with MU-P. This suggest that these cellular lines have more alkaline phosphatases (more content in alkaline phosphatases). Now in terms of sucrose concentration, with AMC substrates, NEL 1 had more activity in M9 and NEL 2 didn't reveled differences between the two sucrose concentrations (except with Met-AMC). With MU substrates, both cellular lines had more activity in M3 than in M9 (except with MU-C for NEL 2). This means that: (1) grow NEL 2 in M3 or M9 appear to not affect the production of proteases and that NEL 2 produce more proteolytic enzymes with 9% (w/v) sucrose; (2) both cellular lines produce more glycoside hydrolases and alkaline phosphates in the presence of a lowest sucrose concentration.

The next objective was to appraise the protein catalytic activity on samples treated with liquid nitrogen, pectinase and glass beads. This assay was performed to confirm the best protein extraction method. The mechanic treatment with a plastic piston was made with NEL 1 and NEL 2 samples grown with M9 and M3. So, this protein extraction is already described and discussed in previous results. For that a fluorescent assay with a set of enzymatic substrates was essayed (Figure 20). Once again, a set of four fluorogenic substrates specific for proteases was used: Phe-AMC, Arg-AMC, Met-AMC and Lys-AMC.

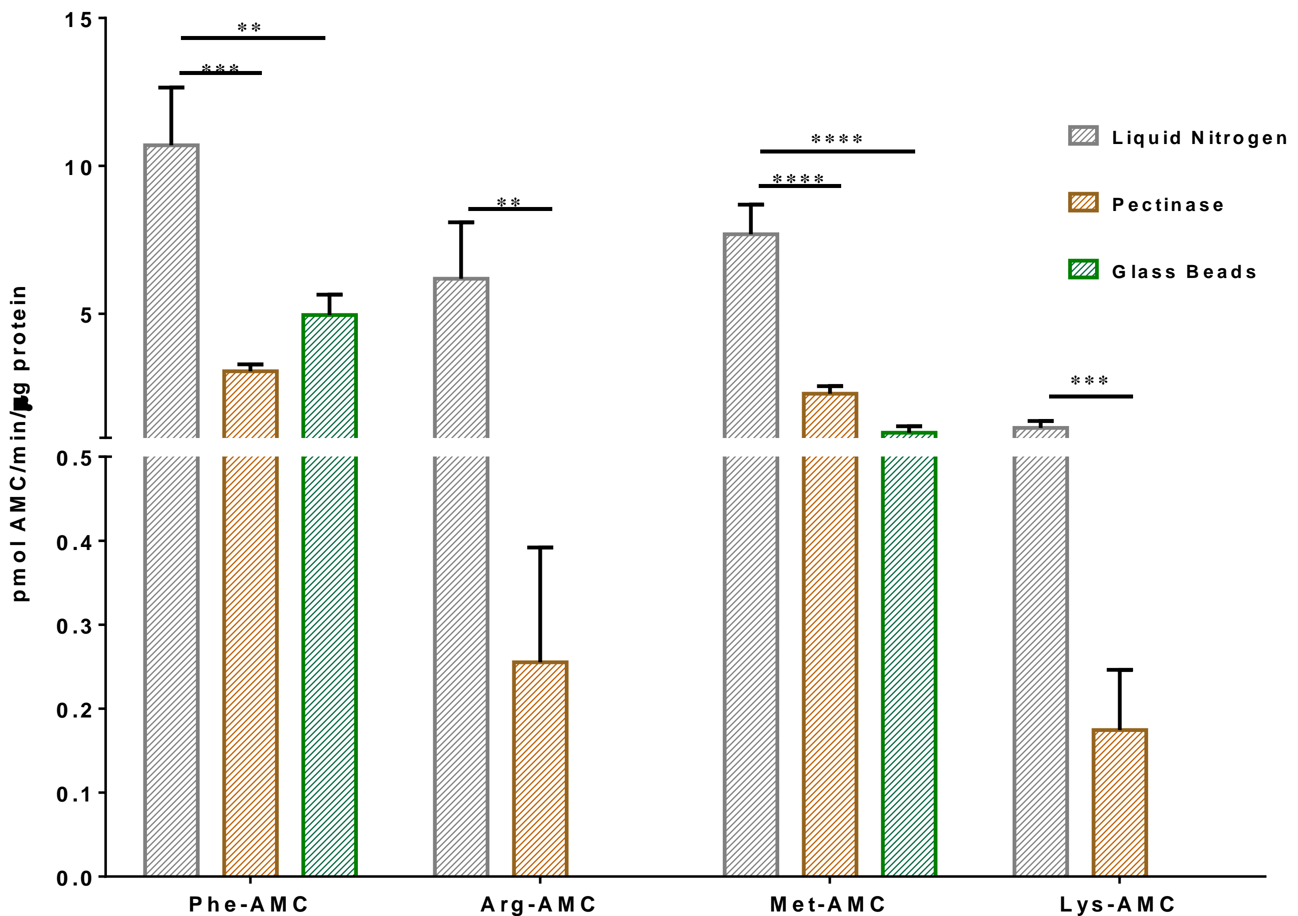

Figure 20. Specific Phe-AMC, Arg-AMC, Met-AMC and Lys-AMC activity of NEL 1 and NEL 2 samples treated with liquid nitrogen, pectinase and glass beads. Data presented as mean ± SD (n=3). Statistical analyze with Tukey test: (p<0.05): **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05.

Enzymatic substrates had activity with all samples, except with Arg-AMC and Lys-AMC substrates for the samples treated with glass beads. For all substrates, samples with liquid nitrogen had the highest specific activities. Within Phe-AMC and Met-AMC, liquid nitrogen samples reveled significant statistical differences when compared to pectinase samples and glass beads samples. Comparing pectinase and glass bead samples, with Phe-AMC, glass beads samples had higher activity, however no statistical difference was found. With Met-AMC pectinase samples demonstrated specific activity superior than glass bead samples, once in terms of statistical significance no dissimilarities were found. Arg-

AMC and Lys-AMC only had activity with samples treated with liquid nitrogen and pectinase and, over again, liquid nitrogen samples showed a higher activity and a significant difference in activity when compared to samples treated with pectinase. An assay with enzymatic substrates having methylumbelliferyl (MU) as fluorogenic group was essayed too: MU-NAG, MU-C, MU-P and MU-G (Figure 21).

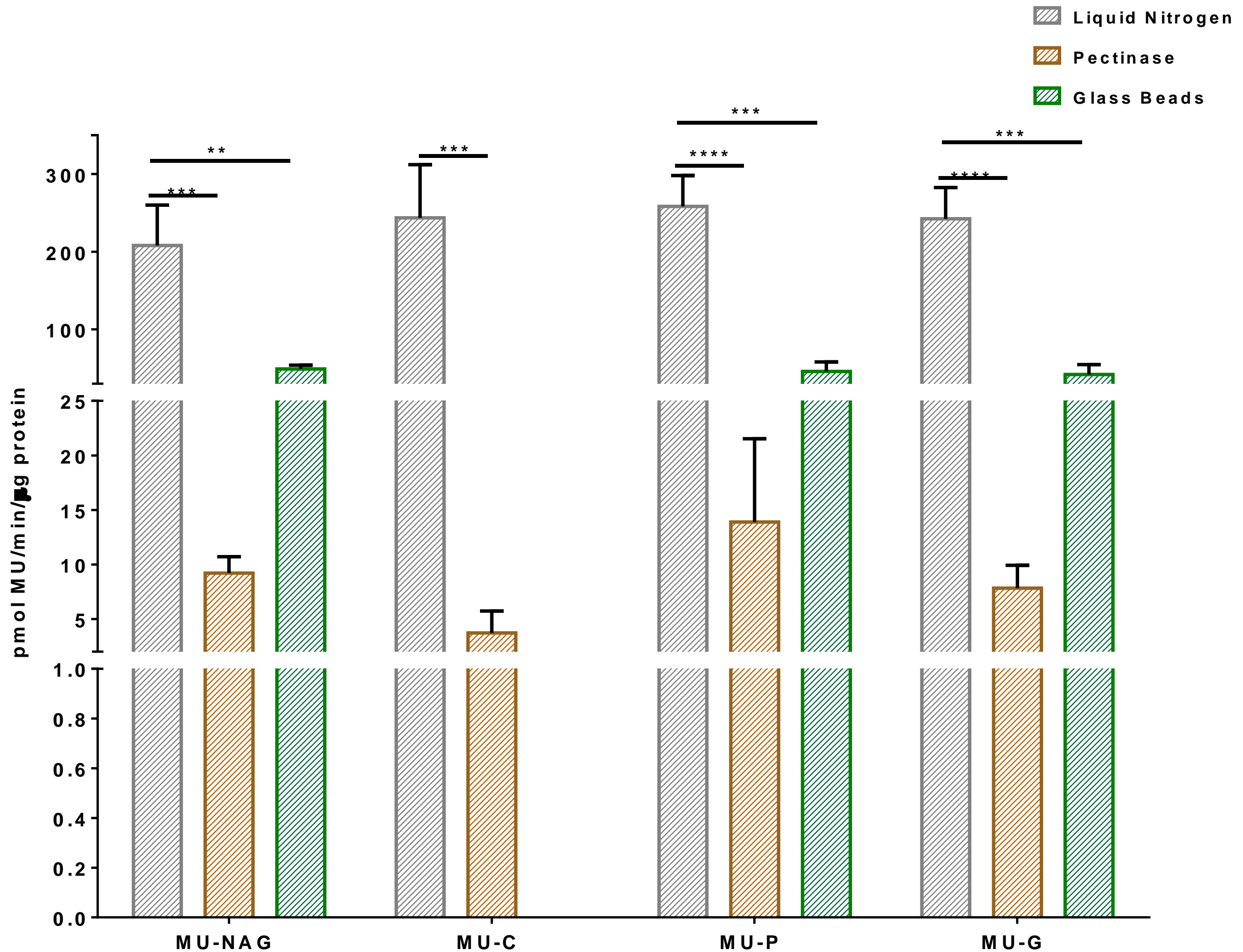

Figure 21. Specific MU-NAG, MU-C, MU-P and MU-G activity of NEL 1 and NEL 2 samples treated with liquid nitrogen, pectinase and glass beads. Data presented as mean ± SD (n=3). Statistical analyze with Tukey test: ($p<0.05$): **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Samples treated with liquid nitrogen and with pectinase exhibited activity for all substrates, but samples treated with glass beads didn't had activity when in contact with MU-C enzymatic substrate. Again, NEL 1 samples treated with liquid nitrogen had major specific activity values. With MU substrates, the activity pattern was quite similar among samples, being samples treated with pectinase the second with high activity and samples treated with glass beads with lowest activity values. Differences between samples were more significant with MU-P and MU-G substrates.

As referred earlier, the focus of this assay was confirm that liquid nitrogen was the best protein extraction method for NEL 1 and NEL 2 cells. Gathering all the results presented in this section, it is evident that mechanic treatment with liquid nitrogen is the most efficacious protocol to liberate protein present in the cells.

The next objective was to assess the protein catalytic activity on NEL 1 and NEL with elicitation. Here, fluorogenic assays are going to be individually evaluated by non-embryogenic cellular line. The first cellular line to be analyzed is NEL 1 (Figure 22). In this sector, the enzymatic assay with fluorogenic substrates specific for proteases encompassed three of the enzymatic substrates referred earlier (Phe-AMC, Met-AMC and Lys-AMC) and more three substrates: Leu-AMC, Ala-AMC and Gly-Pro-AMC. Arg-AMC was removed from the enzymatic assays because almost all samples didn't have activity with this enzymatic substrate. Data are presented in specific activity.

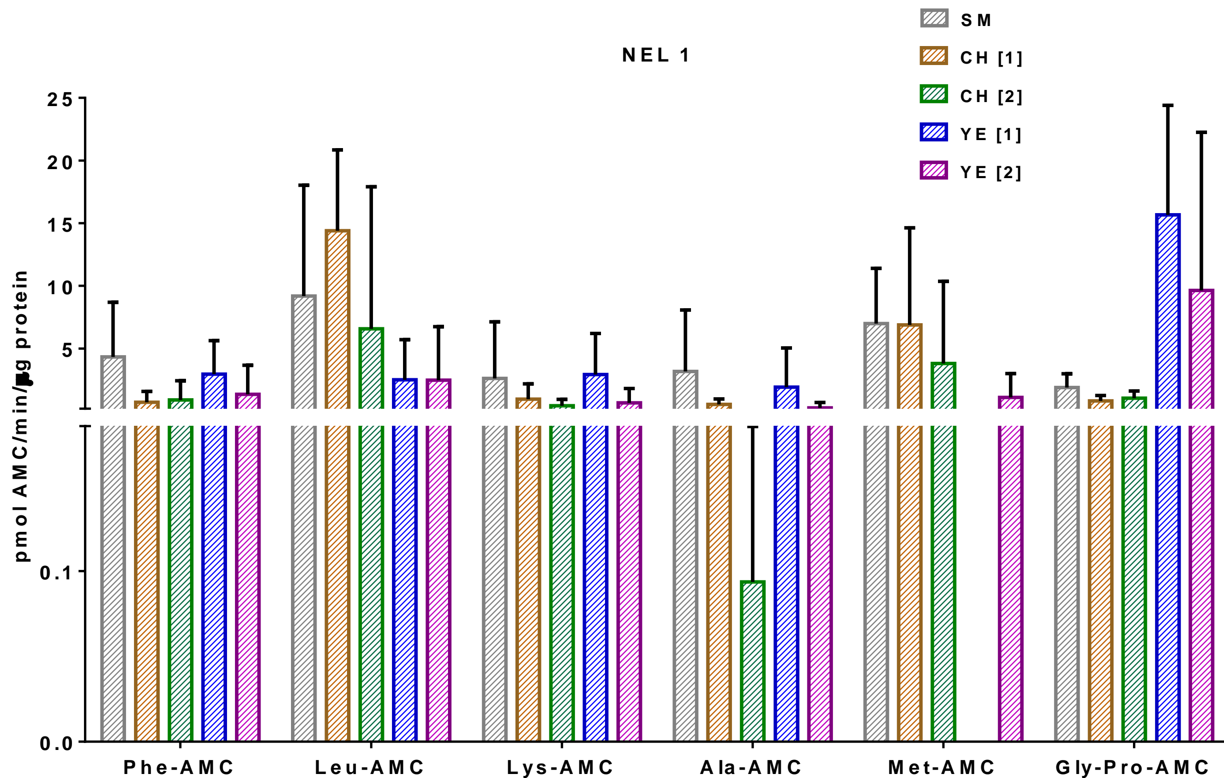

Figure 22. Specific Phe-AMC, Leu-AMC, Lys-AMC, Ala-AMC, Met-AMC and Gly-Pro-AMC activity of NEL 1 in SM, CH [1], CH [2], YE [1] and YE [2]. Data presented as mean ± SD (n=3). Statistical analyze with Tukey test: ($p<0.05$): **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05.

All substrates exhibited activity with all samples, excepted with YE [1] with Met-AMC substrate. As observed, no significant statistical differences were found in samples when compared to the control sample. As demonstrated in NEL 1 SDS-PAGE gel, it samples reveled more protein content in CH [1] and CH [2] samples. However, the zymography gel didn't show any protease activity, this is no bands were found. So, gathering these three results it is possible to conclude that non-embryogenic cellular line 2 produced low amount of proteases, even in the presence of yeast extract and casein hydrolyzed.

Now, in Figure 23 the specific activity for NEL 1 samples with glycoside hydrolases and alkaline phosphatases substrates are represented. The substrates were the same used in earlier results.

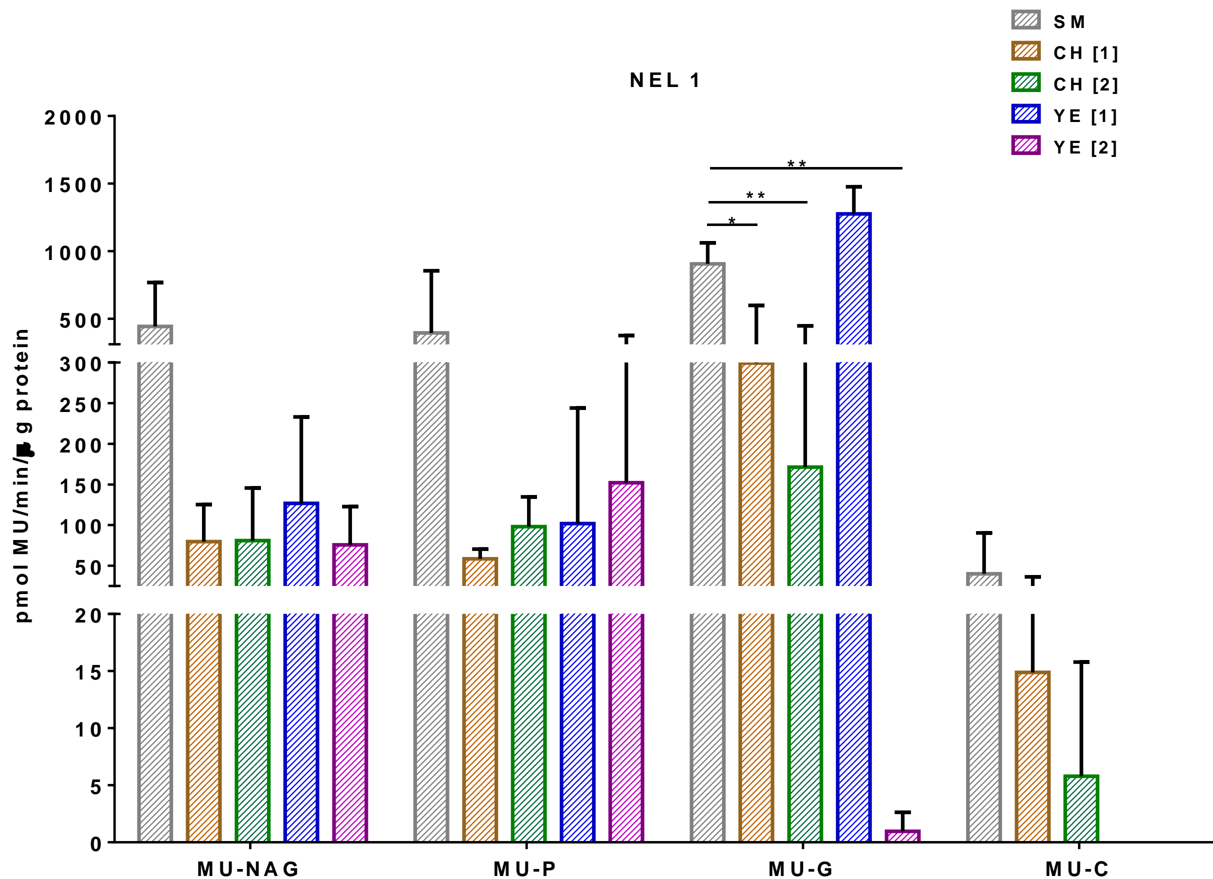

Figure 23. Specific MU-NAG, MU-P, MU-C, and MU-C activity of NEL 1 in SM, CH [1], CH [2], YE [1] and YE [2]. Data presented as mean ± SD (n=3). Statistical analyze with Tukey test: (p<0.05): **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05.

NEL 1 samples exhibited activity with almost substrates, once MU-C only had activity with SM, CH [1] and CH [2]. With MU-G there were significant statistical differences between the control and CH [1], CH [2] and YE [2]. CH [1] and CH [2] had lower activity than the control and YE [2] had higher activity than the control. Once again, samples with elicitors didn't reveled statistical differences with the SM samples. However, with MU-G substrate were differences. So, NEL 1, with and without elicitation, produce low amount of alkaline phosphates and glycoside hydrolases. However, YE [1] samples produce more glycoside

hydrolases specifics for MU-G substrate than the control (SM samples). This may indicate that YE at a concentration of 0,5 g/L may influence the production of glycoside hydrolases specifics for MU-G substrate.

The same evaluations were made for NEL 2. First it was evaluated the proteases activity with AMC substrates (Figure 24).

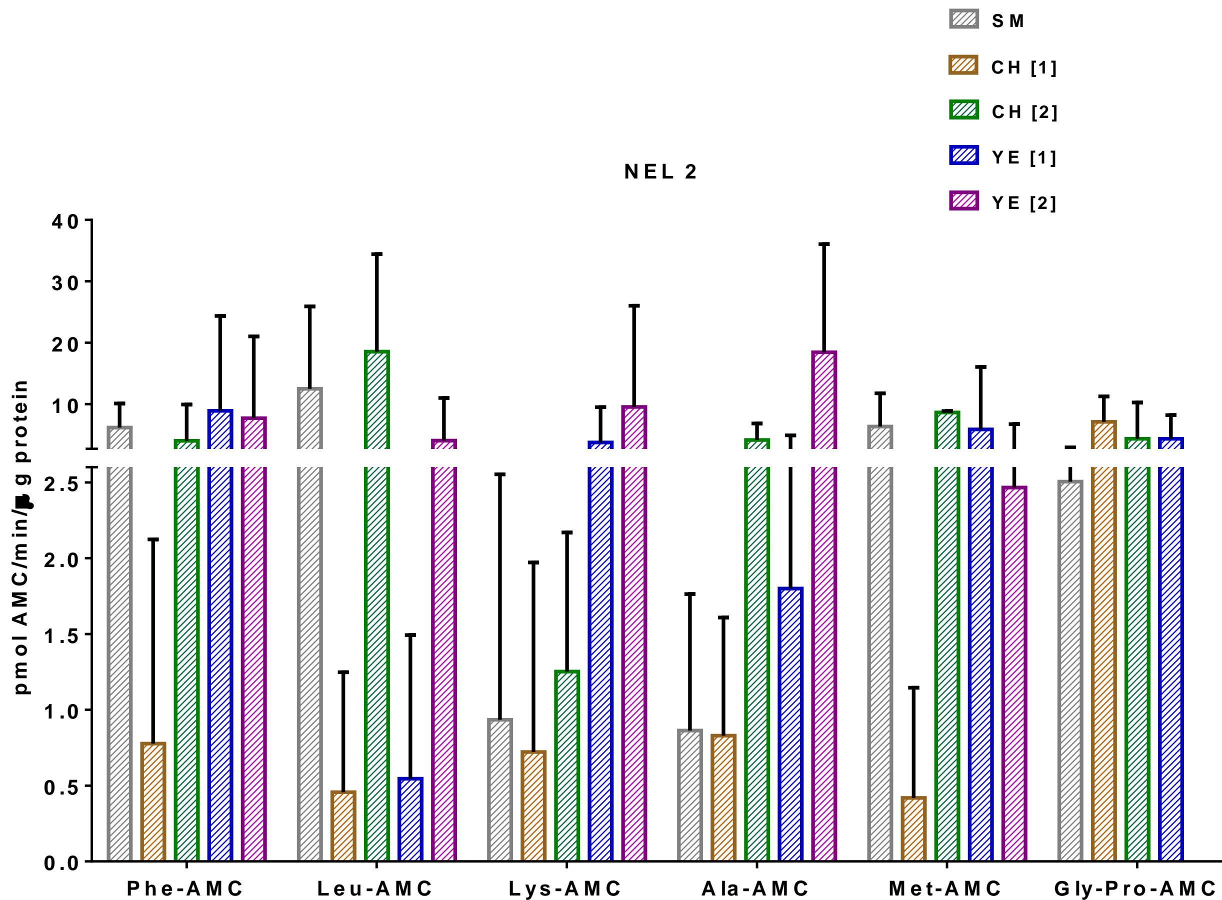

Figure 24. Specific Phe-AMC, Leu-AMC, Lys-AMC, Ala-AMC, Met-AMC and Gly-Pro-AMC activity of NEL 2 in SM, CH [1], CH [2], YE [1] and YE [2]. Data presented as mean ± SD (n=3). Statistical analyze with Tukey test: ($p<0.05$): **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05.

NEL 2 samples exhibited activity with all substrates. However, YE [2] sample didn't showed activity with Gly-Pro-AMC substrate. Once again, no statistical differences were found between elicitors' samples and SM samples. In NEL 2 case, there was bands in all samples when the NEL 2 zymography gel was analyzed. This indicate protease activity in all samples. Moreover, the bands were placed in similar molecular weights, but CH [1] and CH [2] reveled more content in proteases. So, this statistical difference (none) might be related to the heterogeneity in standard deviation caused by the heterogeneity in the biological replicas. However, comparing the protease activity in NEL 1 and NEL 2 samples, it is important to refer that the magnitude order of specific activity was much higher in NEL 2 than in NEL 1.

Now, in Figure 25 is showed NEL 2 glycoside hydrolases and alkaline phosphatases activity.

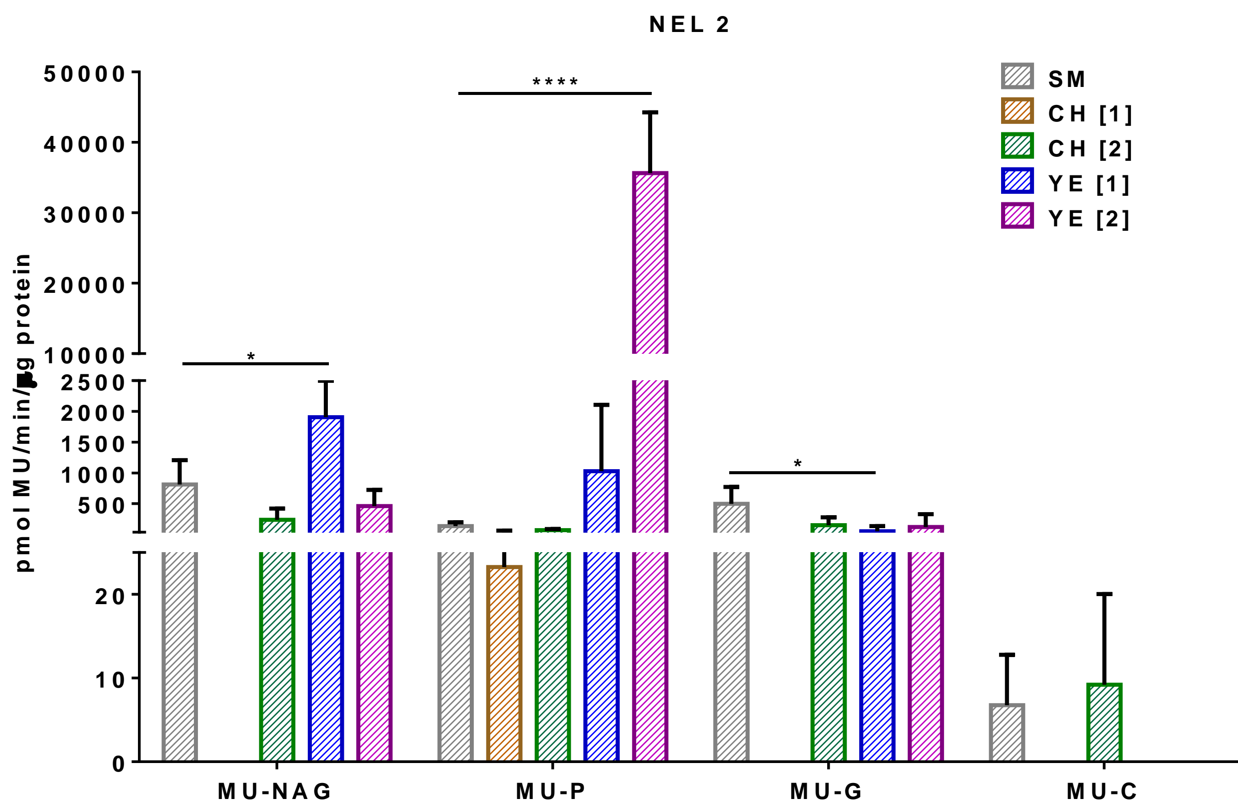

Figure 25. Specific MU-NAG, MU-P, MU-C, and MU-C activity of NEL 2 in SM, CH [1], CH [2], YE [1] and YE [2]. Data presented as mean ± SD (n=3). Statistical analyze with Tukey test: (p<0.05): **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05.

All samples revealed activity with MU-P substrate. CH [1] didn't had specific activity with MU-NAG, MU-G and MU-C substrates and with MU-C only SM and CH [2] samples had activity. Here there were statistical differences: between SM and YE [1] with MU-NAG, having YE [1] a higher specific activity; between SM and YE [2] with MU-P enzymatic substrate, where YE [2] had a superior activity and, again, between sample SM and YE [1] for MU-G substrate, however in this case SM showed a higher specific activity. Here, it is possible to see that YE [1] and YE [2] had higher activities than SM with MU-NAG and MU-P substrates. This means that possible YE at 0.5 g/L influence/enhance the production of glycoside hydrolases specifics for MU-NAG substrates and YE at 1.5 g/L enhance the production of alkaline phosphates. Moreover, comparing the glycoside hydrolases and alkaline phosphatases activity in NEL 1 and NEL 2 samples, it is important to refer that the magnitude order of specific activity was much higher in NEL 2 than in NEL 1.

These fluorogenic assays were performed to evaluate the enzymatic activity in NEL 1 and NEL 2 cells with and without elicitation. Both cellular lines demonstrated differences between SM with elicitation in the production of glycoside hydrolases and alkaline phosphatases. In NEL 1, YE [2] demonstrated a positive influence in the production of glycoside hydrolases specifics for MU-G substrate. In NEL 2, YE [1] demonstrated a positive effect in the production of glycoside hydrolases specifics for MU-NAG substrate and YE [2] showed that is capable to increase the production of alkaline phosphatases specifics. In terms of proteases, both cellular lines didn't demonstrated differences between SM samples and elicitors samples. NEL 1 didn't had bands in it zymography gel but reveled protease activity, which can be because of a low protease quantity. However, the NEL 2 zymography gel reveled more protease activity in CH [1] and CH [2] samples. So, probably, the presence of heterogeneity in the specif activity among samples didn't demonstrated these possible differences between SM and CH [1] and CH [2]. Finally it is important to refer that NEL 2 showed a higher magnitude order of specific activity in both enzymatic assays.

To clarify and investigate these results in the proteases activity, an assay with proteases inhibitors was performed. This assay permits the evaluation of the main classes of proteases in the samples. For that, a set of enzymatic inhibitors specific for proteases were used: Pepstatin A, EDTA, TPCK, TLCK, E-64 and Pefabloc. Instead of adding just one enzymatic inhibitor to the sample, cocktails of inhibitors were performed.

The enzymatic substrate chosen to perform the inhibitors assay was Gly-Pro-AMC. This protease substrate was chosen because for both cellular lines, this substrate had a more homogeneous specif activity for all samples. A control sample was performed for each sample (sample without inhibition). The assay conditions are described in material and methods chapter. The proteolytic activity are expressed in % residual activity. In Figure 26 it is demonstrated the % residual activity of NEL 1 for SM, CH [1], CH [2], YE [1] and YE [2] samples.

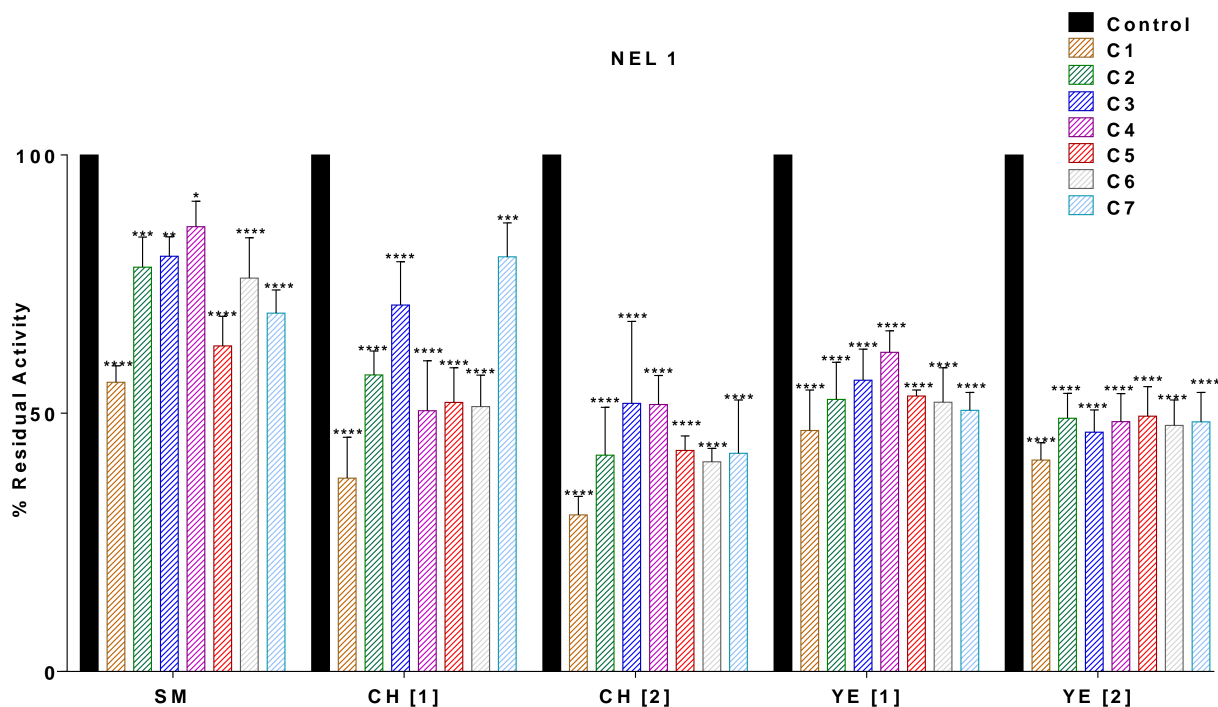

Figure 27. % Residual activity of NEL 1 for SM, CH [1], CH [2], YE [1] and YE [2] samples. C1 – Cocktail with all inhibitors; C2 – Cocktail without Pepstatin A; C3 – Cocktail without EDTA; C4 – Cocktail without TPCK; C5 – Cocktail without TLCK; C6 – Cocktail without E-64; C7 – Cocktail without Pefabloc. Control values were uniformed to 100 %. Data are presented as mean ± SD (n=3). Statistical analyze with Tukey test: ($p<0.05$): **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05.

In all samples, cocktail C1 had the lowest residual activities, which is normal once this cocktail had all the enzymatic inhibitors. All the comparisons were made against the control sample (sample without inhibitors). SM and YE [1] had the highest residual activities with cocktail C4 (cocktail without TLCK) when compared to the control. For sample CH [1], cocktail C7, in which Pefabloc was removed, had the highest residual activity, relatively to the control. Cocktails C4 and C5, cocktails without TPCK and TLCK respectively, were the cocktails with superior residual activities in sample CH [2]. In sample YE [2] there wasn't any enzymatic inhibitor that stand out. In conclusion, cocktails C4, C5 and C7 (TPCK, TLCK and Pefabloc removed, respectively) were the cocktails that exhibited higher residual activities in NEL 1 samples. So, SM exhibited more activity for trypsin-like serine proteases; CH [1] for chymotrypsin-like serine proteases; CH [2] for trypsin-like serine proteases and chymotrypsin and cysteine proteases; YE [1] for trypsin-like serine proteases and YE [2] for trypsin-like serine proteases, chymotrypsin and cysteine proteases and chymotrypsin-like serine proteases. CH [1], CH [2] and YE [2] samples were the ones that demonstrated different main classes of proteases when compared to the control. So, although the fluorogenic assays didn't reveled any differences between samples, here it is possible to see that there are differences in the type of proteases present in each sample. However, comparing the residual activity between samples, SM sample (sample without elicitation) had the highest activities. This may say that yeast extract and casein hydrolyzed potency the production of different classes of proteases when compared to the control.

In Figure 28 it is demonstrated the % residual activity of NEL 2 for SM, CH [1], CH [2], YE [1] and YE [2] samples.

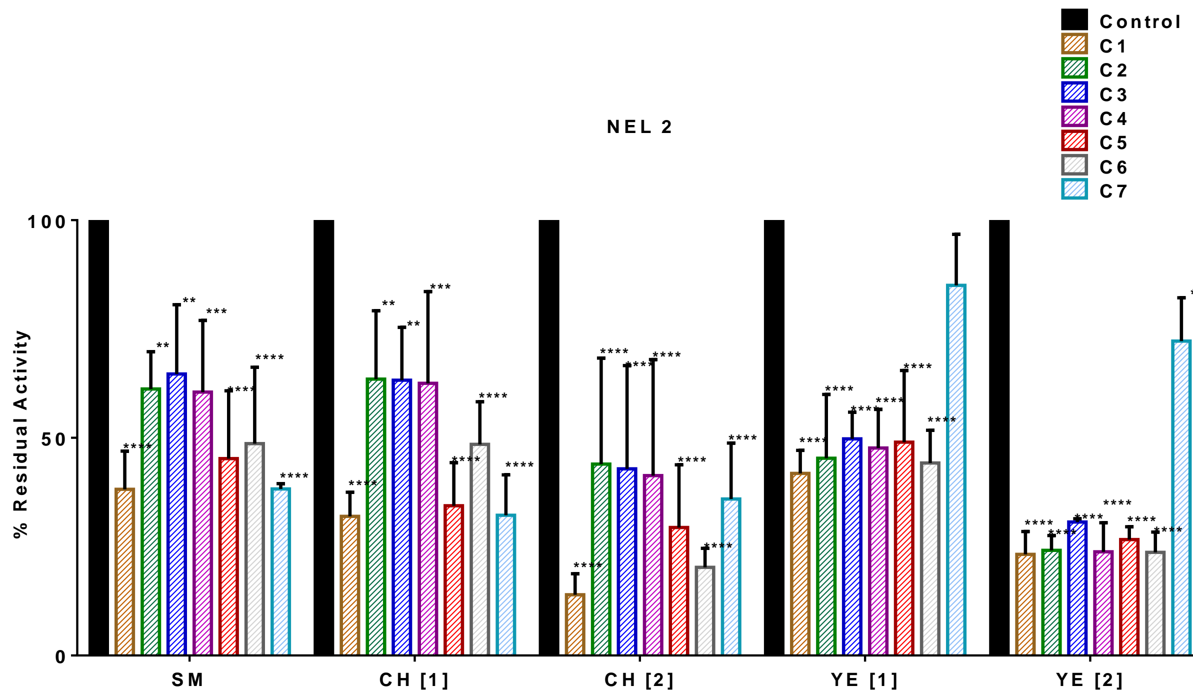

Figure 28. % Residual activity of NEL 2 for SM, CH [1], CH [2], YE [1] and YE [2] samples. C1 – Cocktail with all inhibitors; C2 – Cocktail without Pepstatin A; C3 – Cocktail without EDTA; C4 – Cocktail without TPCK; C5 – Cocktail without TLCK; C5 – Cocktail without E-64; C5 – Cocktail without Pefabloc. Control values were uniformed to 100 %. Data are presented as mean ± SD (n=3). Statistical analyze with Tukey test: ($p<0.05$): **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Over again, in all samples, cocktail C1 had the lowest residual activities, which is normal once this cocktail had all the enzymatic inhibitors. All the comparisons were made against the control sample (sample without inhibitors). In sample NEL 2 SM, cocktail without EDTA (C3) had the highest residual activities when in relation to the control. Samples CH [1] and CH [2] had the highest residual activities with cocktails C2, C3 and C4 (Pepstatin A, EDTA and TPCK removed, respectively). In samples YE [1] and YE [2], cocktail C7 (without Pefabloc) had superior residual activities when compared to the control sample. Here,

cocktails C2, C3, C4 and C7 (Pepstatin A, EDTA, TPCK and Pefabloc) demonstrated major residual activities.

So, SM exhibited more activity for aspartyl proteases; CH [1] for aspartyl proteases, metalloproteases and chymotrypsin and cysteine proteases; CH [2] for aspartyl proteases, metalloproteases and chymotrypsin and cysteine proteases; YE [1] and YE [2] for chymotrypsin-like serine proteases. Here, all elicitors' samples had more main proteases classes than the control. So, casein hydrolyzed and yeast extract potency the production of different proteases when compared to the control.

4. Conclusion

Starting with the influence of the sucrose concentration, for both cell lines (NEL 1 and NEL 2) higher cellular density was achieved with M9 and also a higher intracellular protein content. In terms of enzymatic assays, NEL 1 had higher protease activity with M9, being Phe-AMC and Arg-AMC the preferred substrates, and had more glycosidase hydrolase activity with MU-NAG and MU-C and more alkaline phosphatase activity with MU-P, both in M3. NEL 2 didn't show differences in protease activity between M9 and M3 culture conditions and revealed more glycosidase hydrolase activity with M3 for MU-G and MU-C enzymatic substrates. These are important results to highlight, once the main objective of this work was to optimize tamarillo non-embryogenic cellular lines in terms of products production. So, to grow NEL 1 in M9 reveled more protease production but grow NEL 1 in M3 reveled more glycosidase hydrolases and alkaline phosphatases production. In conclusion, for this cellular line, the sucrose concentration affected the production of different kinds of enzymes, being M9 the sucrose concentration preferred to produce proteases and M3 the concentration preferred to produce glycosidase hydrolases. The production of proteases in NEL 2 was not affected by the sucrose concentrations evaluated, but in terms of glycosidase hydrolases production, 3% (w/v) sucrose was the best sucrose concentration. So, the production of proteases in this cellular line is not affected by the sucrose concentrations evaluated but M3 enhanced the production of glycosidase hydrolases.

The use of liquid nitrogen revealed to be the best approach to extract protein from NEL 1 and NEL 2 cells. This suggest that the cells of both cell line sin study are difficult to lyse, probably having rigid cellular walls. This is very important once the objective is to extract the total intracellular proteins.

Regarding the other task of this work, with the purpose of grow NEL 1 and NEL 2 in contact with biotic elicitors, yeast extract and casein hydrolyzed, and evaluate if these molecules influence the protein production enhance, the first parameter evaluated was the cellular density obtained. NEL 1 demonstrated higher cellular density when it was grown with yeast extract and casein hydrolyzed. However, YE [2] didn't show more cellular density than the control condition. NEL 2 showed, too, higher cellular densities with elicitors. In terms of protein production, NEL 1 samples with CH [1] and CH [2] produce more protein than the control, being 1.5 g/L (w/v) the preferred concentration, and for NEL 2, CH [2] reveled to produce more protein than control samples. So far, in terms of products production, CH [1] and CH [2] are the elicitors that influence more the two cellular lines. In terms of proteases

activity, NEL 1 didn’t exhibited bands in zymography's gel and in enzymatic assays, this cellular line didn’t demonstrate any differences to the control, possibly caused by the low amount of proteases in these cells. NEL 2 samples had more protease activity in CH [2] (zymography’s gel) but didn’t demonstrated any differences to the control in enzymatic assays, caused by the heterogeneity in biological samples. However, in terms of glycosidase hydrolases and alkaline phosphatases production, YE [1] and YE [2] samples had more specific activity than control samples. Moreover, NEL 2 had more protein activity than NEL 1, which means that this cellular line is capable to produce more quantity of proteins/enzymes than NEL 1.

An assay with proteases inhibitors was performed. All samples were compared to a control sample (without elicitation). What was verified was that casein hydrolyzed and yeast extract potency the production of different proteases and the same proteases when compared to the control samples. NEL 1 CH [2] and YE [2] samples produced the same main class of proteases that SM (Trypsin-like serine proteases) and produced too different protease’s classes; CH [1] produce different protease’s classes and CH [2] and YE [1] produced the same proteases when compared to the control. However, in this cellular line, SM samples had more residual activity than the elicitors’ samples. This may say that even the elicitors potentiate the production of different and the same proteases when compared to the control, control samples had more protease activity.

In the case of NEL 2, CH [2] produced the same class of proteases as the control (aspartyl proteases); CH [1] produced the same class of proteases as the control but different classes too and YE [1] and YE [2] produced a different class of proteases. In this case, CH [1], YE [1] and YE [2] had more residual activity for these classes of proteases than the control. This may say that, in NEL 2, the elicitors potentiate the production of different and the same proteases as the control.

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