Use of biotechnological tools as support to domestications and breeding of *Argylia radiata* (L.) D. Don.

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Dedication

To my family, especially my parents Manuel and Cecilia, and my sisters, Gabriela and María José. Thank for to believe in this “project”, to be with me during the complete process, for your support, cares, and help in the hard times. For all the hours on the road and the travels to La Serena to collect plants and seeds.

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Contents

Chapter 1: General introduction, theoretical framework and research project

Use of biotechnological tools as support to domestications and breeding of *Argylia radiata* (L.) D. Don.

1. Abstract

The ornamental plant industry grows year after year and some estimates predict that this market will reach 30 to 60 billion dollars in 2020. This industry has a strong focus on novelty, continuously developing new varieties and introducing species with exotic origins. In this sense Chile has an enormous potential for the generation of new ornamental varieties due to the high degree of endemism of our plant genetic resources, which being domesticated and improved, could become new products for this industry, placing Chile in a position of importance in this market. *Argylia radiata* is one of these plants. This herbaceous perennial, belonging to the *Bignoniaceae* family, has great potential as a new ornamental crop due to its attractive flowers, bluish-green foliage, and place of origin, growing naturally in the aridest desert of the world. The project entitled "Use of Biotechnological Tools as a Support for the Domestication and Breeding of *Argylia radiata* (L.) D. Don.", seeks to implement techniques of tissue culture and polyploidization to advance in the domestication and genetic improvement of this species, in order to be a starting point for the future improvement and development of ornamental varieties of *A. radiata*, while increasing knowledge about this species and enhancing an unknown natural resource, encouraging the study and sustainable use of our valuable plant genetic resources.

2. Introduction

Through history, cultivation of ornamental plants has been associated with advanced civilizations around the world. The use of flowers on rituals and religious celebrations from India, West and South West of Asia, Middle East and Europe it could be traced at less 1.000 years B.C. while, the use of plants for aesthetic reasons was common on ancient Greek and Rome (Horn, 2002).

Ornamental crops industry rapidly grows to global level. Some estimations indicate that global market reaches 27,2 billion of dollar, being The Netherlands the largest producer, with 13,2% of the total market (Aurora IERUGAN, 2010). The expected growth for this sector is 4,2% per year from 2015 to 2020 (Smith, 2015). Other calculations indicate that the ornamental market could be bigger. "Agricultural Market Information Company" (AMI) estimated that on 2016, the consuming of ornamentals by the European Union people reached € 107 per year (IPM, 2017), that means, with a population of 500 million, the ornamental crop market was 53,5 billion of euros on 2016.

On the other hand, the ornamental industry has a strong focus on novelty, it has been characterizing by its large crop diversity and releasing of new species, varieties and cultivars every year. It is estimated that today there are more ornamental crops than all agricultural and horticultural crops combined (Weiss, 2002). The continuous necessity to incorporate new varieties to the market and the economical important that ornamental horticulture is reaching around the world will translate in a promising future to ornamental crop breeding (Horn, 2002). In this sense, Chile has a huge advantage because of its high grade of endemism. 80% of plant species described for Chile are native and more of 52% are endemic (Arroyo et al., 2006), which gives to our country many opportunities to develop highly innovative ornamental crops. This way, the use of biotechnological tools such as tissue culture, polyploidization, hybridization, and somatic embryogenesis, among others, could be useful to the development of new varieties. Regarding this, polyploidization of Chilean native plants could help with its domestication and breeding, valorizing under know genetic plant resources.

2.1. Genus *Argylia*, description and relevant characteristics

As it was mentioned, Chile has unique plant resources which possess a large ornamental potential. This is the case of *Argylia*, that is a genus of *Bignoniaceae* family. It is endemic of

Argentina, Chile, and Perú (Kaderit, 2004). This genus is compounded by 13 species, 10 of them are endemic of Chile: *Argylia adscendens*, *A. bifrons*, *A. checoensis*, *A. conaensis*, *A. farnesiana*, *A. geranioides*, *A. glutinosa*, *A. potentillifolia*. While we share *A. bustillosii* and *A. uspallatensis* with Argentina and *A. radiata* with Perú. Only *A. robusta* is not found on Chile and it has been described as endemic from Argentina (Gleisner and Ricardi, 1969). According to Karedit (2004), the number of chromosomes of this genus is 2n=30.

Argylia is herbaceous plants, which grow as a stumpy rosette. Remarkable characteristic inside this family, because the majority of them are trees, woody bushes and climber plants (Mostafa et al., 2014). Leaf disposition is alternate or opposite, with long petiole. Pubescent lamina, it is palmately or pinnately compound, generally palmately. The root is characterized by to be a reserve structure with a considerable size and usually ramified. *Argylia* plants produce a terminal corymb or raceme inflorescence, except *A. bustillosii* that produces single flowers. Fused campanulate calyx, deeply toothed. Pubescent and showy petals which are fused, forming a trumpet corolla (Gleisner and Ricardi, 1969). That flower shape is a typical characteristic of *Bignoniaceae* family. Flowers are perfect, hermaphrodite and zygomorphic with 5 lobes, 2 upper and 3 lower. Fused stamens. anthers united in an apical way. Superior ovary, which is common in this family, it has abundant ovules with axile placentation. Ovary with 2 carpels. Fruits are dehiscent siliques with persistent calyx. Oblong seeds without wings or with very reduced marginal membranes (Gleisner and Ricardi, 1969).

2.3. *Argylia radiata* (L.) D. Don. Xerophytic species with huge ornamental potential

One of the most representative species of *Argylia* genus is *A. radiata*, or by its common names “Velvet Flower”, “Jote Flower”. This herbaceous perennial plant, native from arid and semiarid areas of Chile, lives from the coast to the intermediate valleys between the Arica Parinacota Region to the Coquimbo Region (around 30°50’ to 26°06’ S), making it one of the most representative plants of "Flowering Desert" (Bianco et al., 1986; Riedemann et al.,

2006). The hard climatic condition where *A. radiata* grows is other of the striking characteristic of this species. It dwells on desertic ecosystems with scarce vegetation of the scrubby type. This area is defined by an average temperature between 13 ° C to 15 ° C and high drought, with annual precipitations that do not reach 79 mm per year, even at the most north they could have only 12 mm per years (Rioseco and Tesser, consulted March of 2017). While the majority of the plants of *Bignoniaceae* family live in tropical and subtropical areas (Mostafa et al., 2014), *A. radiata* grows at the Atacama Desert, the driest desert of the world. This understudied species has an extraordinary potential as ornamental crop due to its green-blue foliage, floral stems of 20 cm to 30 cm long and multiple trumpet flowers. The color range of the flowers goes from white to deep red through tones of yellow, orange and pink (Riedemann et al., 2006). The mentioned characteristics make to *A. radiata* a wonderful candidate to plant breeding by biotechnological tools such as tissue culture, polyploidization, and selection. Through these, it is possible to obtain outstanding individuals which could be new varieties of ornamental crops, taking advantage of an undervalued natural resource and getting, on the future, innovative products with a strong country brand. In this way, it is important to mention that one of the most relevant features for consumers of ornamental plants, it is that new varieties look unique and exotic, which can make them reach high prices for that (Weiss, 2002). On the other hand, the extraordinary capacity of this plant to grow on desert environments, with salty and poor soils, high radiation and huge water stress could develop new ornamental crops that offer new options for xeriscaping. These favor an efficient use of water, especially in zones with drought problems where the current scenery of Global Warming puts water supply at risk.

In this context, polyploidization could be a relevant tool. It has been widely used in plant breeding and according to many authors, it has had an important role over plant

domestication process (Ramsey and Schemske, 1998; Altman and Hasegawa, 2012; Renny-Byfield and Wendel, 2014; Sattler et al., 2016).

2.4. Polyploidization: definitions and importance during domestication and plant breeding

Even when there are differences between authors, polyploidization has been defined as the possession of three or more chromosomes set. This phenomenon is produced during cell division, generating cells, and later, organisms which have a larger number of chromosomes. This can produce in a natural way, by induction with chemicals and also by environmental conditions (Ramsey and Schemske, 1998).

Polyploidization has had a relevant role over plant domestication and breeding. It has permitted to increase the productions of industrial crops such as cotton, sugar cane, wheat, oats, peanuts, soybeans, coffee and tobacco (Rahman and Paterson, 2010). Polyploidization has also had a crucial performance during citrus and banana domestication process (Bradshaw, 2016). Some estimates indicate that 35% to 70% of all plant species are polyploid (Ramsey and Schemske, 1998; Husband et al., 2008; Wood et al., 2009). Meyer et al., (2012) analyzed 203 world important crops and they determined that 64% of them are diploid, 17% are polyploid and close to 19% of these have diploid and polyploid varieties.

Exist two main kinds of polyploids, first ones are autopolyploids, they are defined as plants that have more than 2 complete set of chromosomes, which come from the same species, while allopolyploids have more than 2 set but they come from different species (Bradshaw, 2016). Both types can have an asexual origin, by trough somatic polyploidization, or can be the result of a sexual process, trough non reduced gametes (De Storme and Geelen, 2013).

Somatic polyploids are the result of problems during mitotic cell division, which generate daughter cells with higher chromosomal charge. Eventually, these cells produce polyploid tissues, that generate polyploid organisms (Sattler et al., 2016). Cells fusion is another mechanism of somatic polyploidization, where the resulted cell has the chromosomal charge of both parental cells. Fuentes et al., (2014) achieved, through graft, to get a hybrid between *Nicotiana tabacum* (2n = 48) and *N. glauca* (2n = 24), this hybrid combines the chromosomal sets of both species, getting a new set of 72 chromosomes. This synthetic hybrid, called as *Nicotiana tabauca*, is an allopolyploid generated by somatic hybridization of two diploid cells.

On the other hand, non-reduced gametes are originated by problems during meiotic division, which produce gametes with a higher number of chromosomes (Sattler et al., 2016). De Storne and Geelen (2013) define three main mechanisms to generate diploid gametes:

a) Genome duplication before meiosis. Polyploid cells go through a normal meiotic division, getting, eventually, diploid gametes.
b) Genome duplication after meiosis. Haploid gametes get fusion, generating diploid gametes.
c) Meiotic restitution. Because problems during any of the meiotic divisions, non-reduced gametes are produced.

Meiotic restitution is divided into two types: FDR (First Division Restitution) and SDR (Second Division Restitution). FDR is produced when the first meiotic division goes wrong, whereby, chromosomes crossing over is absence. This way, diploid gametes, with the same genotype of the mother cells, are produced, keeping the level of heterozygosis from parental cells (De Storne and Geelen, 2013). Moreover, in SDR, the first division goes normally, but it is the second one that fails. In this mechanism the crossing over occurs, generating polyploid gametes which are different from their parental cells and losing heterozygosis level of the mother cell.

It has been proposed that the development of non-reduced gametes is an adaptive strategy of the plant because it has observed that in front of adverse conditions such as high temperature, water stress, mechanic injuries, herbivory, and others, the generation of polyploid gametes increases (Ramsey and Schemske, 1998). This augments the genetic diversity of the populations, and therefore, the ability to survive of them.

Aneuploidy is a third kind of polyploidy. It is defined as the addition of 1 or more specific chromosomes, or even chromosomes fragments, but not of the complete set (Chen and Birchler, 2013). This concept can also use to denominate the loss of chromosomes. In both cases, gain or loss, multiple works indicate that these type of mutations have a dramatic effect over phenotype, decreasing the vigor of the plants (Birchler, 2012).

In agriculture exist many examples of polyploid. In the case of autopolyploids we can find tetraploid potatoes varieties (Carputo et al., 2003), triploid bananas (Dantas et al., 1999), hexaploid sweet potato (Roullier et al., 2013), while triticale and Raphanobrassica are examples, widely studied, of allopolyploids (Bradshaw, 2016). The first one is a hybrid between hexaploids or octoploid varieties of wheat with diploid rye (Bradshaw, 2016). The second one is an artificial hybrid between *Raphanus sativus* and *Brassica oleracea*, both species are 2n=18. When they were crossed, fertile individuals, 2n=18, were obtained. In a spontaneous way, F1 population generated polyploid offspring through the action of non-reduced gametes (Sattler et al., 2016). Other examples of allopolyploid are some triploid and tetraploid varieties of apple (Janick et al., 1996); tetraploid varieties of cotton, *Gossypium hirsutum* y *G. barbadense* (Jiang et al., 1998) and allotetraploid crops of coffee tree (Clarindo and Carvalho, 2008).

2.5. Effect of polyploidization over plant development and production

Many effects of polyploidization over plants phenotype have been described. The most studied one is heterosis, also called "hybrid vigor" or "enlarging effect". This phenomenon is able to increase the size of plants organs (Chen and Birchler 2013; Sattler et al., 2016). Heterosis is explained by two reasons: 1) an increase on cell volume produced by DNA rise inside the nucleus; 2) the presence of more copies of genes related with quantitative heritage (Saeidi et al., 2008). Some of the polyploidization effects over plants are:

- A higher biomass production. It has been reported that polyploid plants are more vigorous than their diploid parents. Even, it has been observed that allopolyploids show lager biomass production than autopolyploids, which is caused by higher heterozygosis level on allopolyploids, allowing a better adaptation to the environment (Brichler, 2012).
- Size increasing of plant structures. As it was mentioned, the increase in cell volume and the greater presence of alleles associated with quantitative characteristics can induce the generation of larger leaves, flowers, and fruits. At the same time, they can also increase the flowering period (Chen and Birchler 2013). This is especially important for the ornamental industry.
- Growth rate reduction. Some authors have observed a reduction in the speed of growth of polyploid plants (Kazi et al., 2015a). This could be explained because diploid cells grow faster than tetraploid (Östergren, 1954). Probably, because of the higher amount of DNA that polyploid cells have, this requests more time to be replicated.
- Remediation of deleterious mutations. Because the presence of multiple copies of non-mutated alleles on polyploid individuals, the damaged alleles are not able to express (Sattler et al., 2016).

- Fertility loss. The generation of gametes, embryo and also endosperm are affected by the chromosomic unbalance that show polyploid individuals (Birchler, 2012). This phenomenon is especially observed on triploids, which have additional reproductive barriers because their three sets of chromosomes cannot divide in a correct way during meiosis (Murphy, 2007).
- Fertility restoration. Some polyploids, especially those have odd ploidy, they could have fertility issues. When the ploidy level is increased, doubling the number of chromosomes, it is possible to restore the fertility of those plants (Bradshaw, 2016).
- The hybridization with distant species could be facilitated. The main barrier for interspecific hybridization is the absence of homologous chromosomes which can cross over among them. In polyploids, duplicated chromosomes can cross over between themselves, without to find homologous chromosomes from the other species (Sattler et al., 2016).
- Resistance to stress of biotic and abiotic factors. Polyploid crops show a higher resistance to stress than their diploids counterparts (Sattler et al., 2016). That could be explained by a greater level of heterozygosis showed on polyploids, which give them, adaptive advantages to the environment. Also could be a consequence of the expression of multiple allele copies of genes associated with quantitative heritage.

The mentioned effects have made to polyploidization a powerful tool of plant breeding, allowing the apparition of outstanding individuals which produce bigger fruits, greater biomass, and larger flowers. Over time, these were selected by the first farmers, permitting the domestication of specific crops which laid the basis of human civilization through agriculture.

2.6. Polyploid induction

Polyploid has played a crucial role during plants speciation and domestication (Ramsey and Schemske, 1998; Wood et al., 2009; Chen and Birchler 2013; Bradshaw, 2016; Sattler et al., 2016), becoming in a powerful breeding tool because all the effects that could be induced. The most used strategy to generate polyploids is through induction with inhibitors of the mitotic spindle (Bradshaw, 2016) as colchicine, oryzalin and nitrous oxide.

2.6.1. Colchicine

Colchicine (C22H25NO6; M. W.: 399,437 g/mol) is an alkaloid extracted from wild saffron (*Colchicum autumnale* L.). This was originally used for gout disease treatment, and also for other pathologies as Behcet syndrome. Its use in agriculture, as chromosomal duplication agent, has opened huge possibilities for the plant breeding work (Kazi et a., 2015b). Blakeslee and Avery (1937) were the first ones who reported that applications of colchicine caused a considerable increase in the appearance of tetraploids in plants. Since that moment, this chemist became in the most used polyploidization agent (Bradshaw, 2016).

Colchicine can link to microtubules α and β, inhibiting their polymerization and the formation of the mitotic spindle, which avoid the migration of the chromatids to the cell poles during anaphase (Sattler et al., 2016), which produces a reconstruction of the nucleus with the double of chromosomes (Acquaah, 2007).

Although the use of colchicine is widely disseminated among researchers and breeders, colchicine is highly toxic to plants and animals, and it can cause side effects in treated tissues as mutations and toxicity (Östergren, 1954; Morejohn et al., 1987).

2.6.2. Oryzalin

Oryzalin (3,5-dinitro-N4,N4-dipropylsulfanilamide; C12H18N4O6S; M. W.: 346,36 g/mol), which was originally used as an herbicide, is other of the components widely used for

polyploid induction on plants. Oryzalin, as colchicine, avoids the separation of chromatids due to the inhibition of the mitotic spindle produced by its union with the microtubules (Morejohn et al., 1987). This chemist has demonstrated to be an excellent alternative to colchicine, because it is as effective as colchicine, even to lower concentration. Also, oryzalin is less dangerous for human health (Dunn and Lindstrom, 2007). Some authors affirm that oryzalin is much better than colchicine, because the last one, not only induces polyploidization, but also generates not wanted mutations over the plants (Morejohn et al., 1987).

2.6.3. Nitrous oxide

Nitrous oxide (N_2O; M. W.: 44,013 g/mol), also know it as laughing gas or hilarious gas, is a colorless gas, with a sweet smell and it is slightly toxic. This gas was originally used in the pharmaceutical industry, by its sedative capacity, and also in the car industry.

Östergren (1954) was the first one who demonstrates the capacity of the nitrous oxide as polyploidization agent, after that, it was extensively used on many crops (Okazaki et al., 2005). This gas acts depolymerizing the microtubules of the mitotic spindle, affecting the chromosomes migration to the cell poles (Kitamura et al., 2009). Some of the benefits of nitrous oxide, as polyploidization inductor, are: 1) low toxicity for animals and plants, especially at the induction concentration; 2) as a gas, it is able to spread rapidly in plant tissues, filling the intercellular spaces. This penetration power increases with increasing gas pressure; 3) when the gas is released, this rapidly abandon the plant tissues without leaving residual effects (Östergren, 1954; Kato, 2002).

The gaseous nature of nitrous oxide easily permits to penetrate the tissues, even treating cells that are protected inside plant structures. This is the case of the tulips, in which is not possible use colchicine or oryzalin to generate non reduced gametes, due to the gametogenesis of this species is produced when the floral shoot does not emerge yet from

the bulb, but, with the use of nitrous oxide it is possible to do this kind of treatments (Okazaki et al., 2005).

The application of nitrous oxide over plant tissues is driven inside a high-pressure gas chamber, injecting the gas to between 300 and 600 kPa (Kato, 2002). One of the most common uses of nitrous oxide, as a polyploid inductor, is the treatment of freshly pollinated flowers. This blocks the first mitotic division, producing polyploid embryos and avoiding the chimeras generation (Östergren, 1954; Kato, 2002).

2.6.4. Protoplast fusion

Another mechanism with which it is possible to generate polyploidy is protoplast fusion, also called somatic hybridization. Through this technique, it is possible to develop autopolyploids, fusing cells from the same species. On the other hands, it is also possible to obtain allopolyploids, using cells from different species, which can not combine by cross-pollination. This makes easy the hybridization among distant species (Bradshaw, 2016).

This technique is based on the fuse of non-sexual cells (somatic cells) to which their cellular walls are removed, receiving the name of protoplasts. From that union, a single hybrid cell, called heterokaryon is formed. From this single cell, a new plant could be regenerated through *in vitro* culture (Pensabene, 2009). This technique has been used on several crops, obtaining allopolyploids of tobacco (Carlson et al., 1972), hybrids of *Cucumis* genus (Skálová et al., 2010), hybrids of potato (Bradshaw, 2016), and many others.

Although this methodology has a huge potential to incorporate chromosomes from species distant between them, the implementation of protoplasts fusion needs the development of several biotechnological tools which limit its application such as: an efficient protocol of tissue culture and regeneration from fused cells, callus regeneration, right digestion of cell

walls, correct protocol of fusion, among others. Besides, many of these protocols need to be set by species and variety, forcing the researchers to develop a lot of previous work.

2.6.5. Other factors inducing polyploidy

Although the application of antimitotic agents is the most widely used strategy to generate polyploidy in plants, there are other mechanisms that can induce it. Many of these methodologies were developed before the discovery of colchicine activity in the 1930s (Bradshaw, 2016). One of the most knew is the “Shock Method”, which was developed by Randolph (1932), when he studied mechanism of tetraploid induction in maize. This method consists of subjecting plant structures to abnormally high temperatures, between 40 to 45 °C for periods ranging from 15 minutes to 2 hours. The tetraploid inducing effect of high temperatures had previously been reported in root apex cells of *Pisum* (Sakamura, 1920) and in plants of the genus *Cucumis* (Koshuchow et al., 1928).

According to Ramsey and Schemske (1998) there are environmental factors that stimulate the generation of non-reduced gametes, promoting the subsequent appearance of polyploid individuals. Some of them are: temperatures, especially sudden changes, herbivore, wounds, lack of water, nutritional stress (Grant, 1952), and even diseases. It has been seen that in tobacco plants affected by TMV, the generation of non-reduced gametes increases (Kostoff and Kendall, 1931; Kostoff, 1933). Also, exist genetic factors associated with polyploid generation through non-reduced gametes. It has been observed that hybrid plants tend to form a greater number of non-reduced gametes, probably because the interspecific hybrids have mating problems between their chromosomes, causing imbalances during meiosis and generating diploid gametes (Ramsey and Schemske, 1998). Additionally, it has been observed that in non-hybrid cultures the appearance of diploid gametes increases enormously when selecting the individuals that produce them within the population (Ramsey

and Schemske, 1998), which would indicate that there is an inheritable genetic base that favors the generation of gametes not reduced.

In addition, to those already mentioned, there are other polyploidy inducing agents, such as x-rays (Randolph, 1932; Östergren, 1954); trifluralin and pronomide (amiprophos-methyl). Both compounds act as inhibitors of mitotic use (Morejohn et al., 1987).

2.7. Polyploidization and plant breeding of ornamental crops

The use, induction, and selection of polyploids individuals is a tool that has been observed along ornamental breeding (Horn, 2002; Van Tuyl and Lim, 2003; Kazi et al., 2015a). Most ornamental crops of economic relevance have two interrelated characteristics, they are interspecific and polyploid hybrids (Van Tuyl et al., 2002). This way, polyploidy plays a relevant role in ornamentals development, because many of commercial varieties are polyploid (Horn, 2002).

In the beginning, most ornamentals were diploids, but with the crossing between different species caused by the horticulturists, more hybrids cultivars were developed. Interspecific hybrids produce non-reduced gametes easier, generating allopolyploid offsprings, which were selected and cultivated by their outstanding features (Van Tuyl et al., 2002). This is how interspecific hybridization played a key role in the polyploidization of ornamental crops (Ramanna et al., 2012).

A well-documented example of polyploidization through interspecific hybridization is the case of narcissus. According to Brandham (1986), before 1885 only a few diploid and triploid varieties of narcissus were available in the market (2n=14; 3n=21). In 1887 the first tetraploid variety was introduced and for 1920 a huge explosion in the development and use of polyploid narcissus had occurred. Currently, triploid and tetraploid varieties domain the market of this flower (Van Tuyl et al., 2002). A similar case occurs with freesias, where

tetraploids replaced diploid cultivars around 1950. Other examples are *Begonia semperflorens*, cyclamen, *Primula malacoides*, and geraniums. In all of them, the use and development of polyploids cultivars have had a considerable increase during the last years (Horn, 2002).

How it was mentioned, polyploidization generates multiple effects over plants development. In the specific case of ornamentals, the main results that are searched with polyploidization induction are:

a) *The increase of flower sizes.* A well-known effect of polyploidization is the increment of flowers size, making them more striking (Horn, 2002; Kazi et al., 2015a; Kazi et al., 2015b).

b) *Increasing of vegetative structures.* Although in many ornamentals the main focus is the flowers, it exists a large spectrum of crops which parts of interest are vegetative ones, such as leaves, shoots and also fruits. In those cases, to have larger organs it is a differentiating factor among varieties.

c) *Generate compact growths.* Is has observed on plants such as narcissus, gladioli, and primulas a shortening of internodes as result of polyploidization, generating more compact plants (Horn, 2002). This is a very appreciate characteristic for the ornamental industry, especially in the pot plants segment.

d) *Induction of infertility.* While in many crops infertility is a serious problem, in the case of ornamentals is a desirable feature. In this way, the non-authorized propagation of improved plant materials is limited, and their use as parents is also restricted. Thereupon, the triploids generation gets a huge relevance, because this technique has shown the highest efficiency to develop infertility in plants (Murphy, 2007).

e) *Fertility restitution.* Urwin (2014) achieved to restore the fertility of lavender hybrids (*Lavandula angustifolia* x *L. latifolia*), obtaining viable seeds, and their subsequent progeny.

This can facilitate the generation of hybrid populations, increasing the variability of breeding programs.

f) *Lengthen the flowering period.* It has observed, that in polyploid varieties it is possible to lengthen the flowering period (Chen and Birchler 2013). This is a very desirable feature in ornamentals. It has been reported that somatic autopolyploids of gerbera show a larger flowering lapse than their diploid peers (Gantait et al., 2011).

g) *Facilitation of hybridization among distant species.* The increment in the number of chromosomes allows recombination between duplicated chromosomes, avoiding infertility caused by the absence of homologous chromosomes (Sattler et al., 2016). This way, the variability of the crops can be increased through the incorporation of new genes from other species (Crane and Lawrence, 1952).

h) *Generation of new flower's pigmentations.* It has been described the generation of the "Color Dosing Effect", where through polyploidization is possible to obtain different flower colors (Horn, 2002). Crane and Lawrence (1952) described differences in the color of dahlias with different ploidy level. Shinoyama et al. (2006), mentions changes in the color of polyploid chrysanthemums, which were obtained by induction with colchicine. Even, it has been observed that the variegated in rhododendron flowers are the product of chimeric tissue in the petals. The edge of the petals is composed of tetraploids cells, and the rest of the tissue is diploid (Kazi et al., 2015b). These changes in the flowers color could be explaining by the over-accumulation of pigments, that is produced by multiple copies of genes related to these metabolic pathways.

i) *Generation of deeper green foliage.* In the ornamentals where the part of interest is the foliage, the generation of greener leaves is desirable. It has observed that polyploid varieties of anthurium show deeper greens than their diploid counterparts (Kazi et al., 2015a).

j) *Vase life increase.* Gantait et al. (2011) reported the generation of polyploid gerberas which showed a 5 days longer vase life than their diploid peers.

It is clear that polyploidization has had a huge role during plant domestication and breeding. For that reason, many breeding programs use polyploidization as a common tool (Vainstein, 2002). It has been one of the most used strategies to develop more productive varieties, and in the case of ornamentals, polyploidization has played, and it will follow playing, a relevant role in the development of new varieties.

Polyploidization is a biotechnological tool that could support the work of researchers, nurserymen, and amateur horticulturists of our country. It opens the possibilities to develop new ornamental crops, generated from Chilean phytogenetic resources. The varieties generated with this technique may affect in a positive way the agricultural and economic development of our society.

Thus, the application of techniques of polyploidization induction on *Argylia radiata* could facilitate the domestication of this species, becoming a possible starting point for future breeding programs of ornamentals, and promoting the study and sustainable use of this type of resources.

3. Work hypothesis

Polyploidization of *in vitro* plants of *Argylia radiata* (L.) D. Don, it will facilitate plant breeding of this species by the generation of outstanding individuals.

4. Objectives

4.1. General objective

To use biotechnological tools, as tissue culture and polyploidization, to support the domestication and breeding processes of *Argylia radiata* (L.) D. Don., facilitating the future development of new ornamental varieties.

4.2. Specific objectives

a) To describe *Argylia radiata* biology, such as phenology, reproductive biology, seed germination, and histological aspects.

b) To do chromosomes counting of *A. radiata* through cytological techniques.

c) To do an efficient system of *A. radiata* micropropagation, which ensures the obtaining of clone plants that can be used for all types of tests.

d) To generate polyploid lines, through the application of polyploidy inductors, as colchicine, oryzalin or nitrous oxide, to *A. radiata* microplants.

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Chapter 2: Morpho-Anatomical adaptations of *Argylia radiata* (L.) D. Don to an arid environment

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Morpho-Anatomical adaptations of *Argylia radiata* (L.) D. Don to an arid environment

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Abstract

Argylia radiata is an herbaceous perennial with substantial potential as an ornamental plant. This is a native plant from the Atacama Desert, it can be found in the north of Chile and the south of Peru. This species is part of the "Flowering Desert", which is an unpredictable phenomenon, associated with unusual winter rainfalls that allow the massive blooming of the Chilean Desert. While most plants in family *Bignoniaceae* are originally from tropical and subtropical areas, *A. radiata* lives in very harsh conditions, with average precipitation of around 12 mm per year, a maximum of 34 °C in summer, and a minimum of 2 °C in winter. This extreme habitat makes this species an interesting option to study for its morphological and anatomical adaptations, which have not been described in depth. In this work, optical and scanning electron microscopy of histological samples from different structures were used to describe the plant and to try to understand how *A. radiata* survives in the driest desert in the world.

Keywords

Argylia; Atacama Desert; Xerophytic plant; Plant Anatomy; Plant morphology; Plant adaptation.

1. Introduction

Argylia radiata (L.) D. Don is a perennial plant, part of the *Bignoniaceae* family, tribe *Tecomeae*. The plants of *Argylia* genus are perennials herbs which often grown in rosette form with thick and woody roots. They live in xerophytic environments of Argentina, Chile, and Peru (Gentry, 1992). In Chile, most of the species dwell in the north, between 18°26' to 29°53' S, locating the coast, central valleys, and Andes Mountain. To the south, the species move exclusively to the Andes, around 36°53' S; 71°17' W (Gleisner and Ricardi, 1969). In the case of *A. radiata*, it is native from the Atacama Desert, living at the north of Chile and the south of Peru.

In Chile, *A. radiata* occurs from the costal desert to the intermediate valleys between 30°50' to 18°26' S; 71°05' to 71°15' W. The average temperature range is 14 to 22 °C, with a maximum of 34 °C in summer and a minimum of 2 °C in winter, the driest zones have less than 12 mm of precipitation (Rioseco and Tesser, 2018).

A. radiata is a member of the "Flowering Desert" (Bianco et al., 1986a; Riedemann et al., 2016), a natural and unusual phenomenon that sometimes occurs in the plains and hills of the Coast Range and the intermediate inland depression, both of which are usually devoid of vegetation (Carevic, 2016). This sudden and profuse explosion of plants, especially annuals and bulbous biennials, is associated with the El Niño/Southern Oscillation (ENSO) weather pattern, which brings unusual winter rain to the north of Chile that permits plant

growth (Jaksic, 2001). *A. radiata* become easier to see during this phenomenon, because they have underground structures that sprout quickly when enough water is available.

A. radiata has a huge potential as an ornamental landscape plant, because of its green-blue foliage, multiple floral stems with beautiful and abundant trumpet flowers, and low water requirements. Feuillée (1714), a French botanist, wrote the first description of *A. radiata*, saying, "it was the beauty of this plant that made me describe it". After that, Linnaeus described *Bignonia radiata*, based in the work of Feuillée (Linnaeus, 1753). The flower color ranges from light yellow to deep red through orange and pink tones.

Despite those characteristics, research about this species is limited. Biochemical studies, which detected the presence of different kinds of iridoids, have been done on *A. radiata* (Bianco et al., 1986a; Bianco et al., 1986b; Bianco et al., 1987; Bianco et al.,1991; Bianco et al., 1992). On the other hand, Gleisner and Ricardi (1969) made an exhaustive review of the *Argylia* genus, with a detailed morphological description. However, that characterization was made using only plant materials from herbariums, and underground structures were not analyzed nor were anatomical studies made.

This work presents the study, by optical and scanning electron microscopy of tuberous root, stems, leaves and floral stalk, of the morphological and anatomical adaptations that allow *A. radiata* to survive in the driest desert in the world and how these structures promotes its ornamental potential.

2. Materials and methods

2.1. Plant material collection

All plant materials were collected from nature during the 2015 Flowering Desert event. Above and underground structures were isolated from wild *A. radiata* individuals near

Andacollo, a town located 60 km to the southeast of La Serena, in the Coquimbo Region, Chile (30°12'29" S 71°05'34" W; annual rain of 97 mm; average temperature 16.6°C; 900 m above sea level). Leaves, stems and floral stalks were cut and placed, with wet paper towel, inside plastic bags. Tuberous roots were delicately unearthed and placed on mesh bags. The samples were carefully labeled and stored inside expanded polystyrene boxes to protect them from high temperature and dehydration during transport to the laboratory. Until they were processed, leaves, stems and floral stalks were stored at 3 to 4 °C, while tuberous roots were kept at room temperature.

2.2. Description of tuberous roots

Fifteen tuberous roots were measured and weighed. The smallest, highest, average and variance values for both parameters were determined.

2.3. Sample preparation and analysis

For optical microscopy, all structures were fixed in FAA solution (100 mL L^{-1} 92% ethanol; 20 mL L^{-1} 37% formaldehyde solution; 10 mL L^{-1} glacial acetic acid), inside plastic jars and stored at 3 to 4 °C for one month. After that period, plant material was dehydrated through a series of denatured ethanol washes (Krajnčič, 1989), and dyed with a modified safranin and fast green protocol (Johansen, 1940). Finally, the tissues were placed on a glass slide and covered with a cover slip for their observation under an optical microscope (Olympus CX31 Microscope Series equipped with a camera Evolution™ LC Color, model PL-A662, software Image-PRO® Discovery version 4.5.1.29).

For scanning electron microscopy (SEM), leaves and tuberous roots were fixed overnight at 4 °C in 2.5% glutaraldehyde in phosphate buffer, pH 7.2. The next day, the samples were washed with buffer and dehydrated in solutions of increasing concentrations of ethanol (30, 50, 70, 80, 90, and 100%), each for 10 min. Thereafter, the samples were dried with CO_2 in

a critical point dryer (Quorum Technologies, model K850, UK), and metallized with gold in a sputter coater (SPI Supplies, USA). The tissues were observed using an SEM, model Jeol JSM-6380LV (JEOL, Ltd., Japan) at 20 kv.

To determine the xylem vessel diameter, xylem vessel wall thickness, xylem fiber diameter, xylem fiber wall thickness, leaf thickness, epidermis thickness, stomata density, stomata pore length, stomata pore area, trichome length and trichome density, optical microscopy and SEM images were analyzed using the software ImageJ, version 1.51j8. All parameters were measured 40 times. The smallest and highest values, plus average and variance were determined.

2.4. Starch accumulation

To determine if *A. radiata* stores starch in its tuberous root, a Lugol iodine test was conducted. Lugol solution was prepared by dissolving 20 g of iodine (I_2) and 40 g of potassium iodide (KI) into 1 L of distilled water for 24 h before use. At room temperature, 500 µL of Lugol solution was applied over rhizome slices (around 1 cm thick). After 20 min, the treated samples were analyzed. SEM tuberous root images images were also used to determinate starch presence.

3. Results

A. radiata is a hemicryptophyte that is part of the Chilean "Flowering Desert" that rapidly sprouts on the rare occasions when the Atacama Desert receives winter rains. In a short season, between 6 to 9 months, these plants complete their life cycle, dispersing seeds and then falling into dormancy until the next flowering event, which could take years or even decades. To survive these long periods of drought, *A. radiata* has a large tuberous root, which remains under soil until the right environmental conditions permit the growth of new

shoots. These adaptations have allowed *A. radiata* to live and grow in an arid environment without a regular water supply.

3.1. Root

The tuberous root is one of the most remarkable characteristics of this species. Analysis of the collected samples showed that these structures could reach close to 3 Kg and several centimeters long (Table 1). The tuberous root has a rugous bark, which provides external protection. At its upper portion, it is found the root crown, from which the aerial vertical shoots are developed (Fig. 1a; 2a), which form a rosette of compound leaves. The apical meristem of aerial shoots generates a floral scape with an inflorescence which corresponds to a simple raceme. The root periderm is constituted of multiple layers of cork cells with suberified walls and without cellular contents, derived from a continuous external meristem (Fig. 1d, e, i, k). The cortical parenchyma is composed of small and abundant isodiametric cells, with groups of sclereids arranged vertically (Fig. 1d, e, i, k).

Xylem tissue occupies the largest portion of root volume (Fig. 1f). The xylem vessels are of considerable diameter (around 67 to 150 μm), generally isolated from each other and with lignified walls of scalariform type and simple perforation plates (Fig. 1h). The vessels are inserted into a matrix of parenchyma tissue, that could perform a water accumulation function.

Table 1

Macro and micro measures of *A. radiata* tuberous root.

Item	Smallest Value	Highest Value	Average	Variance (±)
Tuberous root weight (Kg)	0.71	2.93	1.56*	0,68
Tuberous root length (cm)	32.00	76.20	48.99*	12.02

Xylem vessel diameter (µm)	67.01	151.72	101.93**	20.89
Xylem vessel wall thickness (µm)	5.46	20.38	11.57**	13.83
Xylem fiber diameter (µm)	10.05	34.55	21.57**	6.92
Xylem fiber wall thickness (µm)	3.77	7.697	6.77**	1.57

*The average was calculated measuring 15 different tuberous roots.
**The average was calculated measuring 40 different structures or areas.

SEM images showed that the tuberous root of *A. radiata* is not storing starch. Observation by optical microscopy also could not find evidence of accumulated reserves in plant tissues. Lugol tests confirmed these observations, as after 20 minutes the treated slices of tuberous root did not show any reaction.

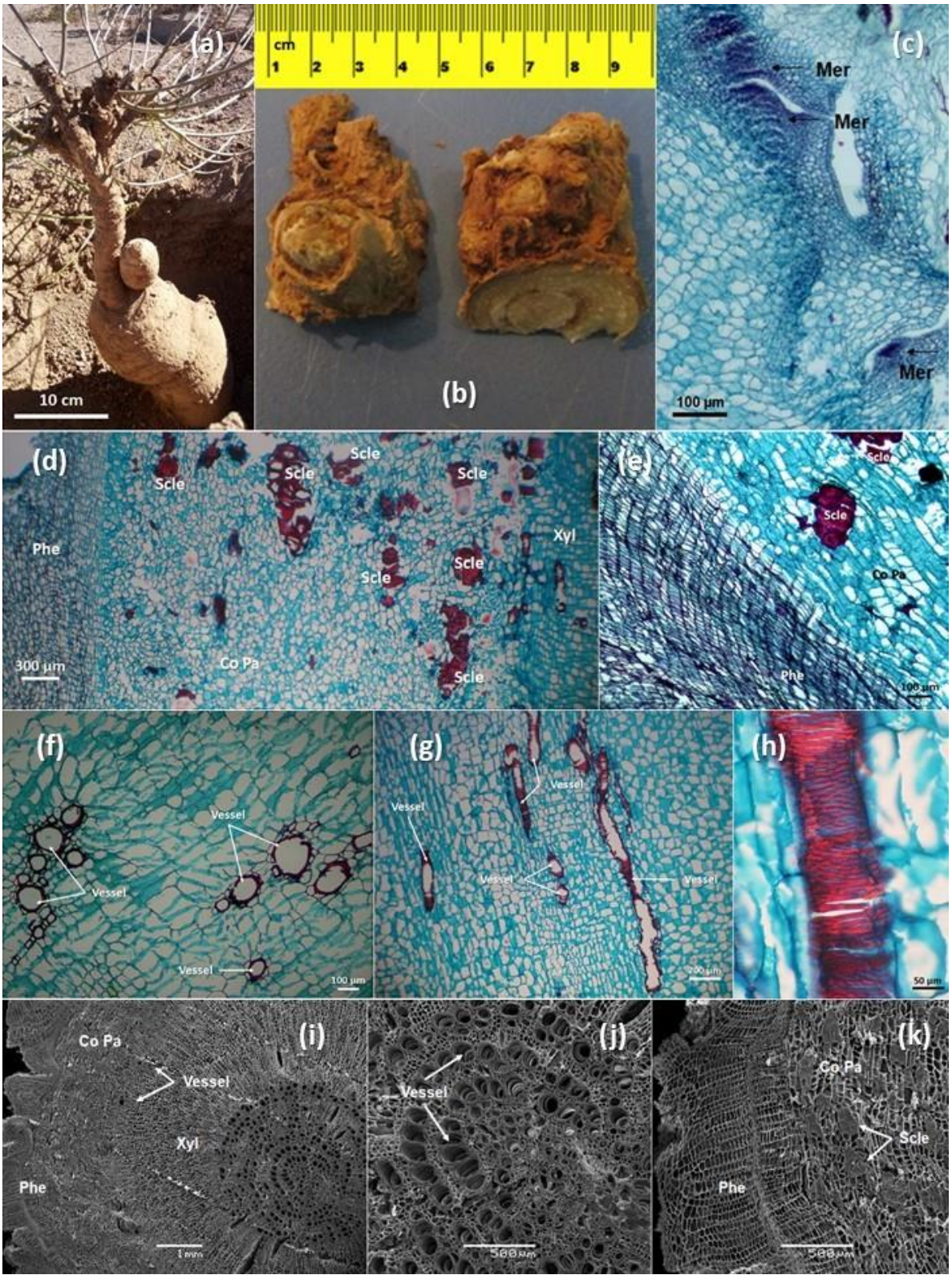

Fig. 1. Disposition of *A. radiata* buds and histological sections of the tuberous root. **a)** Tuberous root, in its upper portion the renewal buds are located. **b)** Detail of root crown. **c)** Cross section of the root

crown, meristematic growth points are labeled (Mer). **d)** Root **l**ongitudinal section. Secondary root growth, starting with a multilayered phellem (Phe), followed by cortical parenchyma (Co Pa), which is formed by isodiametric cells. Sclerenchyma (Scle) arranged vertically, root xylem (Xyl). **e)** Cross section, details of phellem (Phe) and cortical parenchyma (Co Pa). **f)** Root cross section, detail of vessel elements. **g)** Longitudinal section of root, with vessels inserted in parenchyma tissue. **(h)** Longitudinal section of xylem, details of scalariform type vessel. i**)** Scanning electron microscopy of tuberous root cross section. The general distribution of the tissues is observed, phellem (Phe), cortical parenchyma (Co Pa), and xylem (Xyl). **j)** Scanning electron microscopy, central xylem in the root. **g)** Scanning electron microscopy, detail of phellem (Phe) and cortical parechyma (Co Pa), also it is possible to see sclerenchyma insertion (Scle).

3.2. Stems

Primary aerial stems show a monolayer epidermis with multiple trichomes. Under the epidermis, there is a layer of collenchyma and cortical parenchyma. The parenchyma is composed of cells with irregular sizes and shapes (Fig. 2c, d). Grouped collenchyma cells were also present, forming aggregations around the stem periphery, they probably come from protophloems (Fig. 2c, d). Primary vascular tissues encircle a large pith parenchyma, without separate bundles, which has been described in plants of the *Bignoniaceae* family. (Watson and Dallwitz, 1992). No evidence of photoassimilated reserves were observed in the cortical or pith parenchyma.

Aerial stems with secondary growth present 17 to 22 layers of cells with suberified walls, forming a multilayered phellem. Within the cortical parenchyma it is possible to observe sclereids grouped in a similar way to the sclereids found in the cortical parenchyma of the root. The secondary xylem shows large vessels with thick walls (approximately 28 µm), they are inserted on abundant parenchymal cells, similar to the xylem observed in the root. Reserve compounds were not detected in the pith parenchyma. Renewal shoots also grow from the stems along with the secondary growth (Fig. 2b, e, f).

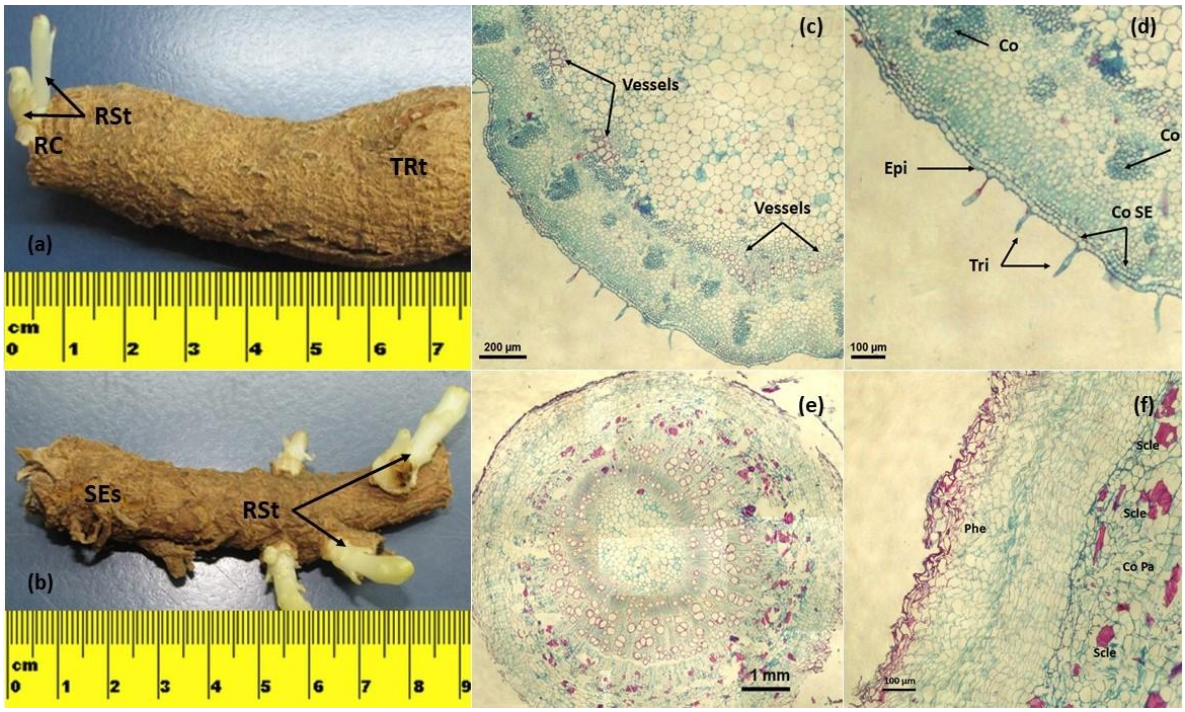

Fig. 2. Stems anatomy. **a)** Sprouted tuberous root (TRt), with renewal shoots (RSt) growing from the root crown (RC), where renewals buds are located. **b**) Sprouted stem (SEs), with renewal shoots growing from a stem with secondary growth. **c)** Cross section of a stem with primary growth, general disposition of tissues is observed. **d**) Detail of trichomes (Tri), monolayer epidermis (Epi), subepidermic collenchyma (Co SE), and grouped collenchyma (Co). **e)** General view of a cross section of a stem with secondary growth **f)** Detail of multilayered phellem (Phe) and the sclereids (Scle) insertion at cortical parenchyma (Co Pa).

3.3. Leaves

The leaves are arranged in a basal rosette, they correspond to a palmately compound leaf with 7 pinnatisect leaflets. Leaf parenchyma has an equifacial structure with spongy parenchyma and numerous chloroplasts (Fig. 3a, b, c, d). Anomocytic stomata were found on both sides; this makes *A. radiata* an amphistomatous plant (Fig. 3a, b, f). Stomata do not show any special characteristics, but the substomatal cavities are notorious (Fig. 3b, c). Leaves have abundant pilosity which is compounded by two types of multicellular trichomes, filiform, and glandular trichomes with a terminal glandular head (Fig. 3g, h). Foliar layer

thickness, epidermis thickness, stomata density, stomata pore length, stomata pore area, trichome length, and trichome density are presented in Table 2.

Table 2

Dimensions of *A. radiata* leaf structures.

Item	Smallest Value	Highest Value	Average	Variance (±)
Foliar layer thickness (mm)	0,30	0,45	0,37*	0,04
Epidermis thickness (µm)	6,32	24,61	13,71*	4,01
Stomata density (stomata/mm^2)	51,64	145,05	88,24*	29,46
Stomata pore length (µm)	5,06	13,24	10,13*	2,63
Stomata pore area (µm^2)	11,80	51,33	29,89*	14,51
Trichome length (mm)	0,03	0,22	0,12*	0,05
Trichome density (trichome/mm^2)	19,72	121,43	58,91*	32,46

*The average was calculated measuring 40 different structures or areas.

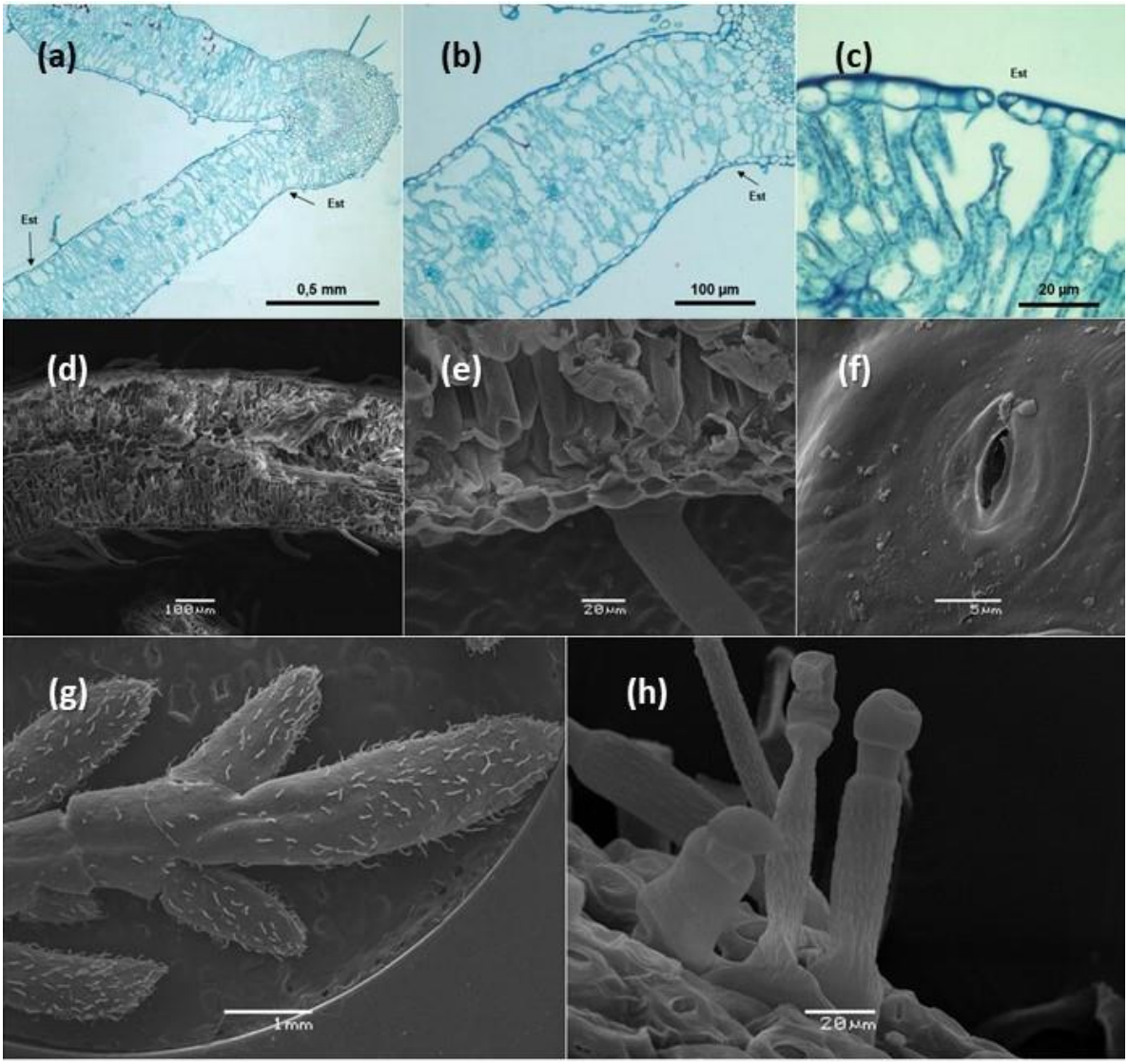

Fig. 3. Microscopic images of *A. radiata* leaves. **a)** Leaf cross section, with stomata (Est) and leaf parenchyma. **b)** Detail of equifacial leaf parenchyma. **c)** Detail of stomata and substomatic cavity. Images **d)**, **e)** and **f)** are from scanning electron microscopy of leaves, and show details of parenchyma, epidermis and stomata, respectively. **g)** General disposition of trichomes at the leaf surface. **h)** Trichome detail, it is possible to see the terminal glandular head of the trichome.

3.4. Floral stalk

This structure is developed from apical buds of the aerial shoots. It shows a monostratified epidermis with abundant pluricellular trichomes. The subepidermic collenchyma surrounds

the entire stalk periphery, forming a ring. Grouped collenchyma cells were also found, located at the soft vertices of the floral stem. Pith parenchyma degraded towards the center of the stalk (Fig. 4).

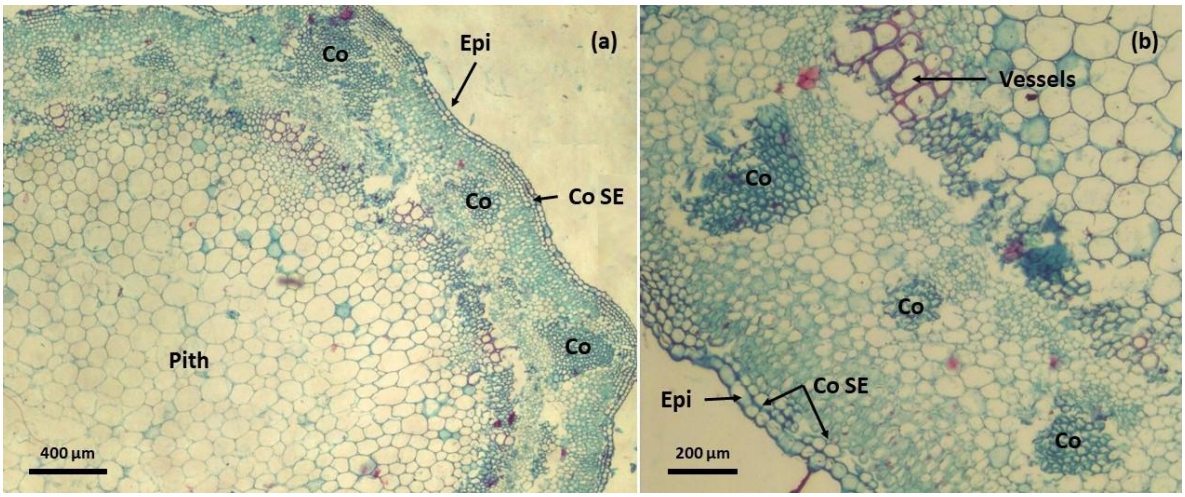

Fig. 4. Cross section of floral stalk. **a)** General disposition of tissues in the floral stem, monostratified epidermis (Epi), subepidermic collenchyma (Co SE) grouped collenchyma (Co), and pith. **b)** Detail of collenchyma tissue (Co; CoSE), and epidermis (Epi). Vessels are also labeled.

4. Discussion

A. radiata is a species in the Bignoniaceae family, adapted to live in the harsh conditions of the Atacama Desert. Its particular morphological characteristics and habitat differentiate it from the rest of the members of its family. It is an herbaceous perennial that grows in a tight rosette, while most Bignoniaceae species are woody vines or trees and live in tropical and subtropical areas (Mostafa et al., 2014).

Xerophytic plants are divided in two main categories, drought resisters and drought escapers. One of the most common adaptations of the second kind is the formation of underground structures, which permit survival during long dry seasons (Cutler et al., 2007). In the case of *A. radiata*, its tuberous root is one of its most notable characteristics, due to

the massive size they can reach (Table 1). This structure allows the plant to remain dormant during the dry season in order to avoid drought conditions. The renewal buds are located at the upper portion of the tuberous root, at or under the soil level, but not too deep. The meristem remains dormant while waiting for optimal conditions, after which they sprout. The shallowness of the buds within the soil allow fast sprouting and growth, and in this way photosynthetic activity can start promptly.

Because of the size of *A. radiata* tuberous roots, it is logical to assume that this structure has a storage function. However, Lugol Test and SEM image analysis were negative for starch. Starch is the most widespread storage carbohydrate in plants (Zeeman et al., 2010), so the fact that *A. radiata* does not accumulate starch is striking. One possibility is that starch reserves were used during the growth of aerial stems. Whereby, at the moment of the plant materials collection, no starch stockpiles were available. Another explanation may be that other kinds of non-structural carbohydrates are being stored. Inulin could be a possibility, as it is widely used as a reserve polysaccharide (Apolinário et al., 2014), and has been isolated from more than 30,000 vegetable products (Wichienchot et al., 2011). Maltose, fructose, and sugar alcohols are also well known as stored carbohydrates (Quentin et al., 2015). Optical microscopy did not show either any evidence of a carbohydrate reserve. The conformation and cellular composition of the cortical parenchyma of the root it is similar to aquiferous parenchyma of cactus, which permit storage of water (Alonso, 2011). This may indicate that the root of *A. radiata* could have an important water storage function rather than a carbohydrate accumulation activity.

The diameter of xylematic vessels of the tuberous root is similar to the observed in the vessels of the roots of other *Bignoniaceae* species. According to the data reported by Ewers et al. (1997), the average diameter of the root vessels of 11 tropical plants of this family was 105.64 ± 28.82 µm, similar to our results (101.93 ± 20.89 µm). The diameters of xylematic

vessels of plants from environments with enough water supply, as tropical and subtropical species, tend to be wider than the vessel diameter of the plants from arid areas, reducing possible problems of cavitation (Olson and Rosell, 2013). *A. radiata* shows a vessels diameter wider than other plants from dry sites. Gibson (1973), measured the vessel diameter of 19 species of *Cactaceae* family, and its average diameter was 66.78 ± 15.30 µm. On the other hand, Carlquist (1966) evaluated the vessel diameter of 167 plants from dry and desert locations, determining that the average diameter of their xylematic vessel were 39 and 34 µm respectively.

Primary growth stems are born from the root crown, which is located at the cauline portion of the tuberous root, and also from stems with secondary growth. The herbaceous tissue has a monolayered epidermis, followed by a subepidermic collenchyma layer and grouped collenchyma cells. Collenchyma is a live cell tissue that is considered a specialized type of parenchyma with thicker walls. These give mechanical support to the stem, and its peripheral position, next to epidermis, is highly characteristic (Evert, 2006). In this kind of shoot, it is possible to see a large pith, which could be acting as a water reserve. Large, living cells, such as pith parenchyma cells, can store and release water in case of deficit (Holbrook, 1995).

Stems with secondary growth show a structure similar to the root, with a multilayered phellem, which gives protection against external factors. The abundant aquiferous parenchyma, which was also observed in the tuberous root, could act as a water reservoir. It is well known that water is abundant on parenchymal cells, which act as water reserve (Evert, 2006).

The *A. radiata* leaf has deeply dissected edges. This reduction in leaf area is a common adaptation of plants from arid areas, which helps them retain water (Fahn, 1985; Farooq et al., 2012). Leaves are anatomically adapted to maximize the efficiency of photosynthesis. It

was possible to observe an equifacial structure, with spongy parenchyma with well developed substomatal cavities on both leaf sides. Compact mesophyll, with reduced intercellular spaces, has also been reported as a xeromorphic character (Cutler et al., 2007; Dzomeku, 2012), but this kind of tissue is not observed in *A. radiata* leaves. Apparently, during evolution of this plant, it has favored photosynthetic process over water losses. Proof of this is absence of typical adaptations to a dry environment, like sunken stomata, thick cuticle, epicuticular waxes, and thick outer walls in the epidermal cells. All these adaptations limit gaseous exchange, reducing the photosynthetic capacity of the plants.

Stomata seem to follow a similar direction. *A. radiata* has amphistomatous leaves, which are more common in plants from arid environments (de Boer et al., 2016). It has been reported that plants with amphistomatous leaves are more successful in habitats with high light intensity (Mott et al., 1982). This is because in these areas, internal CO_2 concentrations can limit the photosynthetic rate; plants compensate for that restriction by developing stomata on both side of the leaves (Camargo and Marenco, 2011). On the other hand, the number of stomata on the leaves is relative high, showing a similar or even higher density (88,24 ± 29,46 stomata/mm^2) than some other plants from the wetter areas of Chile, such as *Peumus boldus* (115,60 stomata/mm^2), *Gomortega keule* (100,07 stomata/mm^2), *Laurelia sempervirens* (81,25 stomata/mm^2), and *Laureliopsis philippiana* (62,50 stomata/mm^2) (Barrera y Mesa, 1992). Despite this, it is not possible to confirm that the photosynthetic activity is higher in a plant with a greater stomatal density, because many internal and external factors affect photosynthetic rates (El-Sharkawy et al., 1985). Maybe *A. radiata* has developed amphistomatous leaves and a considerable stomatal density to compensate for the reduction on its leaf area.

Leaf pubescence is a common adaptation in desert zones, since it reduces leaf temperature and wind speed, decreasing water loss (Ward, 2008). It has been reported that some

species can develop leaf pilosity under extreme conditions, even when it is not a common feature in their families (Cutler et al., 2007). The observed trichome density in *A. radiata* was similar to other herbaceous perennials from desert climates (Fahmy, 1997). There was a high variance between samples (58,91±32,46 trichomes/mm^2), possibly because leaf pubescence is the result of the interaction of different factors, such as environmental conditions, genetic predisposition, and even leaf age.

During the analysis of the microscopy images, some trichomes were observed to have glandular terminal heads. These glandular heads of the trichomes could contain some kind of protective substances. During Flowering Desert events, the arthropod populations experience an explosive increase in response to greater availability of food sources, which could mean a high herbivorous pressure (Vidiella et al., 1999; Cepeda-Pizarro et al., 2005). Previous studies found different kinds of iridoids in the aerial parts of *A. radiata* (Bianco et al., 1986a; Bianco et al., 1987; Bianco et al., 1991). These compounds have been described as a powerful tool against herbivory, because they impart a bitter taste and reduce the nutritional value of plant tissues (War et al., 2018). *A. radiata* may produce irioids to protect its photosynthetic surface in order to take advantage of the short period of good growth conditions. Further research is necessary to determine if the glandular heads of trichomes contain iridoids that serve as a defense against herbivory.

The floral stalk cross-section showed the presence of collenchyma. This tissue gives mechanical support to plant organs, possibly providing enough rigidity that the *A. radiata* floral shoot can support itself up to 80 to 100 cm in height. This tall, strong stalk is a desirable property in ornamental cut flowers.

5. Conclusions

Most of the plants from *Bignoniaceae* family live in environments with adequate water supplies. In the case of *A. radiata*, the presence of leaf adaptations that prioritize gas exchanges (equifacial spongy parenchyma and amphistomatous leaves) and the absence of adaptations that reduce water loss but limit photosynthetic rates (thick serous layers, multistratified epidermis, sunken stomata). Furthermore, the diameter of the xylematic vessels of the root is wider than the observed diameter in the vessels of plants from arid zones. These are signals that *A. radiata* prioritizes photosynthesis process over water loss. In the same way, the large size of its tuberous root leads to the hypothesis that the survival strategy of *A. radiata* is to maximize photosynthesis during the short period with adequate water in order to accumulate the largest quantity of reserves and to complete its life cycle so that it can once again resist a long drought, but there was no evidence of reserves found on root tissue. On the other hand, the cortical parenchyma of the root and stems is similar to aquiferous parenchyma of cactus, so maybe the tuberous root has a water reserve function. In this way, *A. radiata* has adaptations that promote photosynthesis but do not store carbohydrates. Perhaps the photoassimilates are used to complete the reproductive cycle and to generate biomass during the short time in which Atacama Desert can support plant growth. It is necessary to further study the types of photoassimilates that are not Lugol reactive, that *A. radiata* produces and stores. Moreover, the morpho-anatomical characteristics of *A. radiata* that allow it to grow in desert areas make this plant a good candidate for development as an ornamental plant for use in xeriscaping.

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Chapter 3: Control of shoot tips necrosis during *Argylia radiata* (L.) D. Don micropropagation

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Control of shoot tip necrosis during *Argylia radiata* (L.) D. Don micropropagation

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Abstract

Argylia radiata is a herbaceous perennial plant, native from the north of Chile and the south of Perú. *A. radiata* has a great ornamental potential. Therefore, its micropropagation was evaluated. Previous assays showed that cytokinin supplementation on the growth medium improves the multiplication rate of *A. radiata* but it produces shoot tip necrosis over the microplants. To avoid this effect, changes in growth medium were tested, evaluating their effect over damage level, number of shoots, multiplication rate, plant height (cm), fresh weight (g), dry weight (g), and water content (%). The use of WPM as basal medium showed the best effect, it reduced the damage level and improved the multiplication rate. Also, IBA supplementation treatments were effective to reduce necrotic damage. However, 0.05 and 0.1 mg L^{-1} of IBA significantly decreased the multiplication rate while 0.01 L^{-1} showed a better multiplication rate than the control medium. The other treatments showed no significant improvements over the damage level of microplants.

Key words: *Argylia*; micropropagation; shoot tip necrosis; growth medium; *Bignoniaceae*.

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Authors contribution statement

PMT made all field and lab works. He also wrote the first manuscript of this research and made the necessary corrections.

MG gave all the scientific support for the research structuration, supervising all field and lab works. She also helped to write the first manuscript and checked the different version of this research.

Introduction

Argylia radiata (L.) D. Don is a perennial plant, member of the *Bignoniaceae* family and native from arid and semiarid areas of the North of Chile, between the Atacama and Coquimbo regions (Gleisner and Ricardi 1969). It is also part, and a representative species, of the “Blooming Desert” (Bianco et al. 1986a; Riedemann et al. 2016), phenomenon defined like the appearance of a great number of plants that cover plains and hills, from the coast to the intermediate depression, which are usually devoid of vegetation (Carevic 2016). This understudied species can be a new option for ornamental crops due to its green-blue foliage and multiple floral stems with 20 to 50 beautiful trumpet flowers. The color range of the flowers goes from white to deep red through tones of yellow, orange and pink. The extremely

hard conditions where these plants grow could make this species an interesting option for low usage of water landscaping. Besides, working with *A. radiata* is an opportunity to recognize the value of unused native genetic resources.

As part of the domestication process of *A. radiata*, micropropagation could be a powerful tool to help its breeding program because tissue culture allows a rapid propagation of outstanding phenotypes. On previous trials, it was possible to micropropagate plants of *A. radiata* on Murashige & Skoog medium (MS) (Murashige and Skoog, 1962) without growth regulators, generating *in vitro* lines of this plant. However, the observed multiplication rates were not satisfactory. For that reason, the supplementation with different kind of exogenous cytokinins was evaluated (kinetin, zeatin and BAP). The addition of 0.5 mg L^{-1} of BAP in the growth medium increased the multiplication rates significantly. Nevertheless, its use also generated shoot tip necrosis (STN), reducing the quality of the plant material. STN is a physiological disorder observed in the micropropagation of many plants (Barghchi and Anderson 1996). It could be produced by different factors such as calcium and boron deficiency, vitamins and growth regulator dearth, ethylene, hyperhydricity, agar concentration, explant age, successive subcultures (Bhojwani and Dantu 2013), endofitendophytic contamination (Liu et al. 2005), and even the over supplementation with gibberellic in some cases (Durand-Cresswell et al. 1982). To avoid or reduce this disorder, changes in medium components such as calcium supplementation, an increase of agar concentration, IBA addition and changes of basal mediums were tested during *A. radiata* micropropagation.

Materials and Method

A previous line of microplants was used for all experiments. That line was obtained from plant material gathered during the 2017 Blooming Desert. Herbaceous shoots were

collected from Bahía Inglesa, North of Chile (27°07'32" S; 70°48'53" W). Plant material was protected with wet paper towels inside plastic bags during the transport and it was kept at 4 to 7 °C until it was processed. The initiation process start dipping the shoots on fungicide solution (Captan 20 g L^{-1}) for 30 min. Then, they were passed through 70% (v/v) ethanol solution and washed in 20% (v/v) solution of commercial bleach during 20 min. After that, the material was rinsed three times with sterile water for 5 min. The explants were cut, eliminating all damaged tissue. Finally, nodal sections were planted in MS basal medium (Duchefa Biochemie B.V., catalog code M0222). It was supplemented with 30 g L^{-1} of sucrose and 6.5 g L^{-1} of agar, pH was adjusted to 5.8. After 8 weeks, when lateral shoots were completely expanded, they were cut and isolated to fresh medium. The obtained microplants were kept on *in vitro* condition during 5 months. They were transferred to fresh medium each 5 weeks.

The medium used as control was obtained from previous trials, where the addition of exogenous cytokinins was evaluated. Its composition was MS basal medium, including vitamins, supplemented with 0.5 mg L^{-1} of BAP, 6.5 g L^{-1} of agar, 30 g L^{-1} of sucrose, and pH 5.8. The first batch of treatments corresponds to calcium supplementation. MS basal medium, with its vitamins, was supplemented with three concentrations of calcium nitrate ($Ca(NO_3)_2$). This way, 0.3, 0.6 and 9.0 g L^{-1} were tested. The second group of trials was related to agar content. The agar concentration has been also reported as a possible cause of STN. Control medium had 6.5 g L^{-1} of agar, whereby, 8.0 and 10.0 g L^{-1} of agar were used as treatments. Regards to auxin enriching, it was driven adding three concentrations of IBA 0.01, 0.05 and 0.1 mg L^{-1}. Control medium was kept IBA free. Finally, the last group of treatments was run testing different basal mediums. The control is based on MS medium, whereby, Lloyd & McCown medium (WPM) and DKW/Juglans medium (DKW), with their respective vitamins, were evaluated (Lloyd and McCown, 1980; Driver and Kuniyuki, 1984).

Both mediums were supplied by Duchefa Biochemie B.V. (WPM catalog code M0219 and DKW catalog code D0247). For all treatments, the amount of BAP, sucrose concentration and pH were maintained.

Under flow cabinet, 60 explants were used for each treatment, putting 10 plants per glass jar. After 5 weeks inside growth room (23 ± 2 °C, 40% of humidity and 16 light hours) the damage level, number of shoots, multiplication rate and plant height (cm) of 50 plants were evaluated. Damage level was defined with a scale from 0 to 5, where 0=no damage; 1=soft damage, less of 25% of the tissue shows damage; 2=medium damage, 25% to 50% of the plant's tissue shows necrotic injuries; 3=severe damage, 50% to 75% of the tissue has necrotic damage; 4=serious damage, over 75% of the plant's tissue shows necrotic injuries; 5=dead plants, it is not possible to obtain explants from them. Fresh weight (g), dry weight (g), and water content (%) of 10 plants were also determined. Dry weight was obtained weighting the microplants after being dehydrated at drying oven for 48 hours at 70 °C. Water content was calculated using fresh and dry weight.

All data were analyzed with Kruskal-Wallis test.

Results

The results show that some of the changes in the growth mediums significantly improved the quality of the microplants, while other modifications got a worse response than the control medium. The assessment of the different treatments was principally obtained based on the occurrence of necrotic injuries. Despite this, other parameters, related to the efficiency of *A. radiata* micropropagation, were also evaluated (Table 1, 2, 3, 4).

Table 1 Results of *in vitro* growth parameters of *A. radiata* cultivated on growth medium supplemented with different amounts of calcium nitrate.

Treatment	Damage level[1,2]	Number of shoots[2]	Multiplication rate[2]	Plant height (cm)[2]	Fresh weight (g)[3]	Dry weight (g)[3]	Water content (%)[3]
Control	2.15[a]	5.71[a]	3.00[a]	4.20[a]	0.83[ab]	0.07[a]	91.53[a]
0.3 g L^{-1} $Ca(NO_3)_2$	2.92[b]	4.75[b]	1.80[b]	4.38[a]	0.66[b]	0.08[a]	89.45[a]
0.6 g L^{-1} $Ca(NO_3)_2$	2.74[b]	5.89[a]	2.72[a]	5.03[b]	0.68[b]	0.05[a]	92.48[a]
0.9 g L^{-1} $Ca(NO_3)_2$	2.79[b]	5.58[a]	2.56[a]	5.01[b]	0.91[a]	0.08[a]	94.26[b]

Different letters indicate significant differences between groups ($p<0.05$).

[1]Numeric scale from 0 to 5, where 0 = not damage, plants without necrotic tissue and 5 = dead plants, it is not possible to obtain new explants from them.

[2]Averages calculated with a repetition of n=60

[3] Averages calculated with a repetition of n=10

Calcium supplementation got a worse response that the control medium. The necrotic damage showed by the microplants from the treatments was greater than the injuries observed in the control medium (Fig. 1b, c, d). The number of shoots and multiplication rate neither were better than the control response. Even, the supplementation with 0.3 g L^{-1} of $Ca(NO_3)_2$ was significantly worse than the control medium for both parameters. About plant height, 0.6 and 0.9 g L^{-1} of $Ca(NO_3)_2$ improved the height of the plant.

Regards fresh weight, dry weight and water content (%), no differences between the control and the treatments were observed.

Table 2 Results of *in vitro* growth parameters of *A. radiata* cultivated on growth media with different agar concentration.

Treatment	Damage level[1,2]	Number of shoots[2]	Multiplication rate[2]	Plant height (cm)[2]	Fresh weight (g)[3]	Dry weight (g)[3]	Water content (%)[3]
Control (6.5 g L^{-1})	2.15^{ab}	5.71^{a}	3.00^{a}	4.20^{b}	0.83^{a}	0.07^{a}	91.53^{a}
Agar 8 g L^{-1}	1.77^{a}	6.03^{a}	3.30^{a}	4.35^{ab}	0.78^{a}	0.18^{a}	83.85^{a}
Agar 10 g L^{-1}	2.29^{b}	5.57^{a}	2.75^{a}	4.71^{a}	0.65^{a}	0.05^{a}	91.70^{a}

Different letters indicate significant differences between groups ($p<0.05$).

[1]Numeric scale from 0 to 5, where 0 = not damage, plants without necrotic tissue and 5 = dead plants, it is not possible to obtain new explants from them.

[2]Averages calculated with a repetition of n=60

[3] Averages calculated with a repetition of n=10

The damage level was not significantly different between the agar concentration treatments and the control medium (Fig 1e, f). Nevertheless, a slight reduction of necrotic injuries was observed when 8 g L^{-1} of agar was used.

No differences, between the control and the treatments, were observed in the number of shoots, multiplication rate, fresh weight, dry weight, and water content.

About plant height, the treatment of 10 g L^{-1} produced taller plants than the control medium (4.71 vs 4.20 cm).

Table 3 Results of *in vitro* growth parameters of *A. radiata* cultivated on growth medium supplemented with three concentrations of IBA.

Treatment	Damage level[1,2]	Number of shoots[2]	Multiplication rate[2]	Plant height (cm)[2]	Fresh weight (g)[3]	Dry weight (g)[3]	Water content (%)[3]
Control (IBA free)	2.15[c]	5.71[a]	3.00[b]	4.20[b]	0.83[a]	0.07[a]	91.53[a]
IBA 0.01 mg L^{-1}	0.75[b]	4.37[b]	3.87[a]	5.18[a]	0.67[a]	0.05[a]	91.91[a]
IBA 0.05 mg L^{-1}	0.85[b]	3.65[b]	3.06[b]	5.29[a]	0.74[a]	0.06[a]	91.66[a]
IBA 0.1 mg L^{-1}	0.42[a]	2.32[c]	2.29[c]	4.41[b]	0.64[a]	0.06[a]	90.16[a]

Different letters indicate significant differences between groups ($p<0.05$).

[1]Numeric scale from 0 to 5, where 0 = not damage, plants without necrotic tissue and 5 = dead plants, it is not possible to obtain new explants from them.

[2]Averages calculated with a repetition of n=60

[3] Averages calculated with a repetition of n=10

IBA treatments produced a better response over STN than the control medium. It is possible observe a trend of damage reduction with the increase of IBA concentration (Fig 2c, d, e).

Regard the multiplication rate, the supplementation with 0.01 mg L^{-1} improved this parameter respect to the response showed by the control medium. The multiplication rate produced by the supplementation with 0.05 mg L^{-1} was not different from the rate observed in the control medium. While 0.1 mg L^{-1} of IBA induced a significant reduction in the multiplication rate.

About plant height, 0.01 and 0.05 mg L^{-1} of IBA produced higher plants than the control medium. The supplementation with 0.1 mg L^{-1} of IBA wasn't significantly different than the control medium. Probably, this effect was the result of apical dominance that is induced by the application of exogenous auxins, which reverses its response in high concentrations as 0.1 mg L^{-1}.

Fresh weight, dry weight and water content didn’t show differences between the control medium and IBA treatments.

Table 4 Results of *in vitro* growth parameters of *A. radiata* cultivated on different basal mediums.

Treatment	Damage level[1,2]	Number of shoots[2]	Multiplication rate[2]	Plant height (cm)[2]	Fresh weight (g)[3]	Dry weight (g)[3]	Water content (%)[3]
Control	2.15[b]	5.71[a]	3.00[b]	4.20[b]	0.83[a]	0.07[a]	91.53[ab]
WPM	0.15[a]	5.85[a]	7.09[a]	4.54[b]	1.12[a]	0.07[a]	93.59[b]
DKW	2.67[b]	4.72[a]	2.45[b]	5.33[a]	1.03[a]	0.12[a]	82.56[a]

Different letters indicate significant differences between groups ($p<0.05$).

[1]Numeric scale from 0 to 5, where 0 = not damage, plants without necrotic tissue and 5 = dead plants, it is not possible to obtain new explants from them.

[2]Averages calculated with a repetition of n=60

[3] Averages calculated with a repetition of n=10

In the case of the trials of basal medium, the best results were observed on WPM medium, which produced a significant reduction of necrotic injuries (Fig. 2b). Regard the incidence of STN during *A. radiata* micropropagation, the effect of WPM was the best of all treatments. No differences were observed between the control and DKW medium (Fig 1g).

The number of shoots didn't show any differences between the treatments, but the multiplication rate showed an increase in WPM medium. This was caused by the reduction in STN, that permitted a higher quantity of healthy tissue was available to be used as explants.

About plant height, DKW medium produced taller plants than the control and WPM mediums.

The fresh weight, dry weight, and water content didn't show any differences between the control and the treatment mediums.

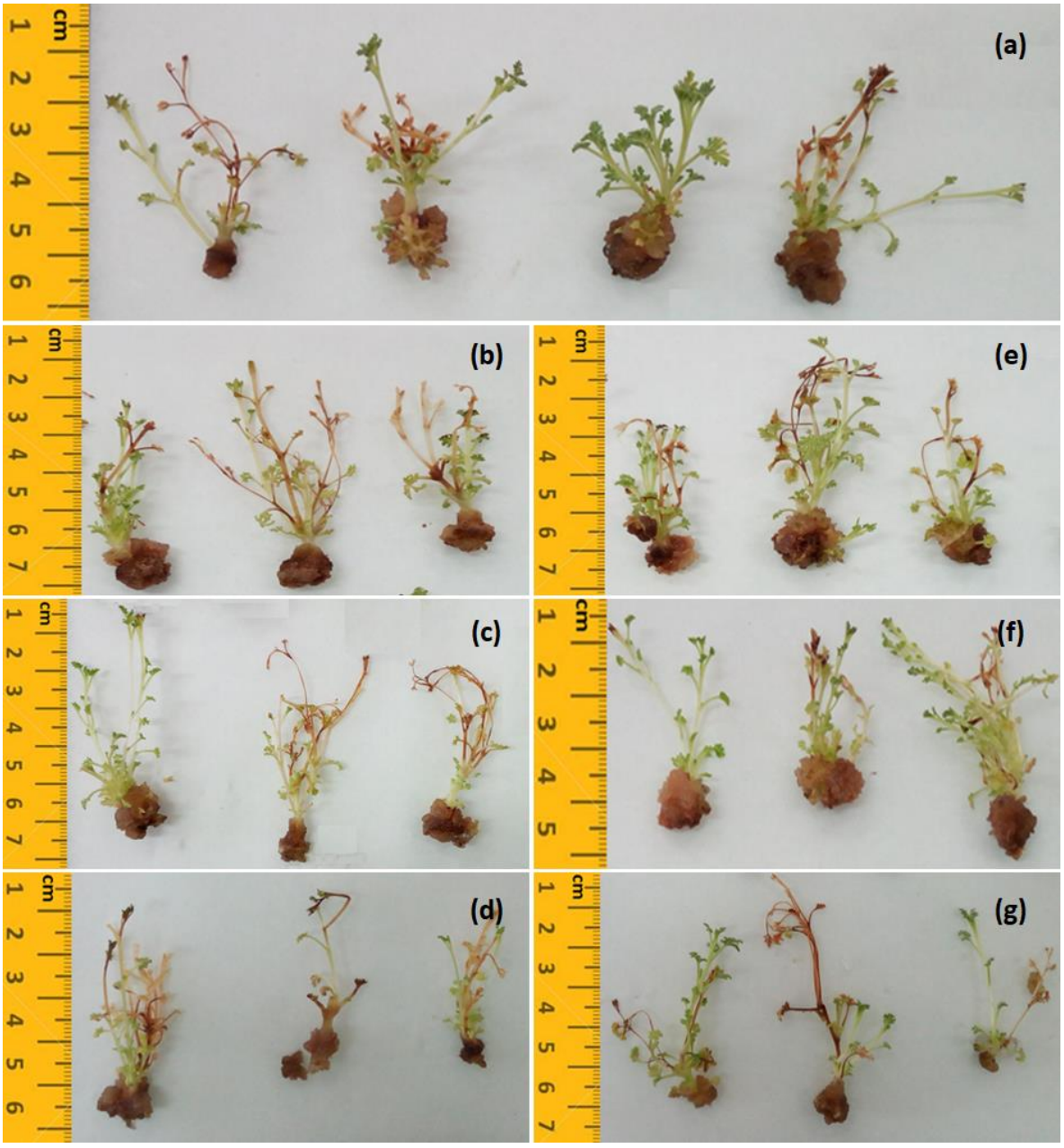

Fig. 1 Develop of STN on different modifications of *A. radiata* growth medium. **a** Control medium, MS basal medium, supplemented with 0.5 mg L^{-1} of BAP, 30 g L^{-1} of sucrose, and pH 5.8. **b**, **c, d** Calcium supplementation treatments, 0.3, 0.6, and 0.9 g L^{-1} of $Ca(NO_3)_2$ respectively. **e, f** Changes in agar concentration, 8 and 10 g L^{-1} respectively. **g** Plants cultivated on DKW basal medium.

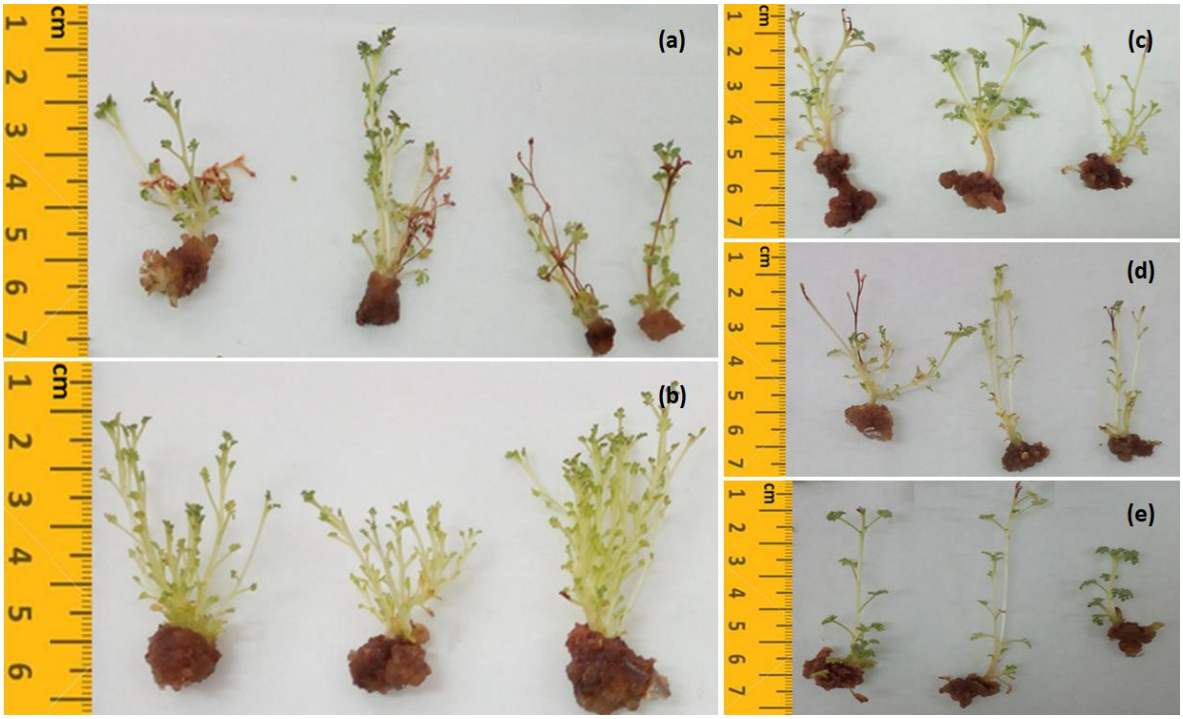

Fig. 2 Reduction of shoot tips necrosis over microplants of *A. radiata* cultivated on different modifications of growth medium. **a** Control medium, MS basal medium, supplemented with 0.5 mg L^{-1} of BAP, 30 g L^{-1} of sucrose, and pH 5.8. **b** Use of WPM medium as basal growth medium. It is possible observe the absence of necrotic injuries when this medium is used. **c**, **d**, **e** IBA treatments, 0.01, 0.05, and 0.1 mg L^{-1} respectively. A reduction of necrotic injuries and a decrease in the number of lateral shoots were observed.

Discussion

The occurrence of necrotic damage during plant micropropagation is commonly associated to nutrient deficit, specially calcium and boron, but actually it is the result of a complex set of factors (Bairu et al. 2009). Deficiency of vitamins and plant regulators, mineral toxicity, ethylene, the age of the explants, vitrification and agar concentrations have been described as possible causes. Therefore, there is no universal method to control it (Thakur and Kanwar 2011). In the case of *A. radiata*, previous experiments showed that the addition of cytokinins

worsens STN problems. This effect has been reported in different species (Bhojwani and Dantu 2013). But also, low levels of this hormone has been described as one of the possible causes. During the micropropagation of apricot and *Trichosanthes dioica*, the occurrence of necrosis injuries has been associated with a reduction in the internal levels of cytokinins (Pérez-Tornero and Burgos 2007; Kishore et al. 2015). Even, continuous subcultures could produce necrosis over microplants of different species (Singha et al. 1990; Rios Leal et al. 2007).

Barghchi and Anderson (1996), reported that calcium supplementation reduces STN in *Pistacia vera* micropropagation, but in our case, the addition of calcium nitrate got worse response than the control medium. This may be an effect of the increase on nitrate level, which could be toxic for *A. radiata*.

Moreover, in many cases more than a deficit, the problem might be related to nutrient absorption and translocation. There is a boundary in the amount of calcium that is possible add to the medium, after which a toxicity problem could develop due to the over-accumulation of calcium because of its limited absorption (George et al. 2008). On the other hand, the intake of calcium by microplants is preferably located on its basal and middle area, while apices accumulate fewer amounts, producing damages over this tissue (Miel et al. 2014). In microplants of *Harpagophytum procumbens*, which develop STN, a supplementation of five times higher than normal calcium concentration didn't show any decreasing of necrotic injuries (Bairu et al. 2009).

Agar treatment didn't produce significant differences over necrotic damage of the microplants. Despite this, 8 g L^{-1} showed a mild reduction of the STN, making it a good option to be used as the standard agar concentration to *A. radiata* micropropagation. On the other hand, 10 g L^{-1} of agar induced an increase in the height of the plants. An increment in the agar concentration limits the movement of medium components, including growth

regulators (Cameron 2008). This could be producing less absorption of BAP, increasing internal levels of auxins. Hence, more apical dominance and a gain in plants height. This is a similar effect to the produced by the application of exogenous auxins.

IBA treatments reduced, in a significant way, the necrotic damage of the vitroplants, but the shoots regeneration was also reduced. This is an expected result because of IBA supplementation induces apical dominance and reduces lateral shoots emission (Bernabé-Antonio et al. 2012; Kyte et al. 2013; Kaviani and Negahdar 2017). Despite this, 0.01 mg L^{-1} produced a higher multiplication rate than the control medium. The decrease in the necrotic damage, induced by the IBA supplementation, compensated the reduction in the number of shoots and it allowed to obtain a better multiplication rate. Less necrotic damage is translated in a higher amount of healthy tissue available to be used as explants. The rest of the IBA treatments didn't improve the multiplication rate.

Some authors propose that the addition of exogenous auxins in the growth mediums reduces shoot necrosis because of the formation of root apices which produce cytokins, raising the internal levels of this hormone. Parallel, that increment of cytokinins elicits the formation of lateral shoots, increasing the levels of auxins. This synergistic reaction improves levels and ratios of endogenous plant hormones and increases the microplant growth during *in vitro* cultures (Bairu et al. 2009). Also, the addition of exogenous auxins could regulate, by itself, the internal amount of hormones, promoting cell division and plant growth. In our case, it wasn't observed an increase of *in vitro* roots development, so probably the effect of IBA over *A. radiata* microplants is related to the improvement of internals ratios of plants hormones.

WPM basal medium produced the better effect over *A. radiata* microplants, reducing, in a drastically way, the necrotic damage of the tissues and improving the multiplication rate. This medium has been successfully used on many species, generally woody plants

(Ďurkovič 2003; Šedivá et al. 2013), but some herbaceous also have been successfully micropropagated in this medium (Bantawa et al. 2011; Jia et al. 2011; Gomes Pêgo et al. 2014). In the case of *Taxus baccata* micropropagation, the use of this medium produces a significant reduction of STN (Ewald 2007).

That necrosis reduction could be explained by the amounts and sources of essential elements which are contributed by WPM basal medium.

Calcium deficit has been described as one of the most common causes of necrotic injuries during plant micropropagation (George et al. 2008). According to George et al. (2008), while MS medium provides calcium in the form of calcium chloride, WPM contributes a higher amount mostly from calcium nitrate (5.98 versus 6.01 meq L^{-1} of Ca^{+2}). Despite this, according to the obtained results with calcium supplementation trials, it is possible that other components of the basal medium are playing a more relevant effect over the necrotic damage and the vitroplants growth.

One possibility is the major concentration of chloride ions supplied by MS medium, 5.98 versus 1.3 L^{-1} of Cl^{-} from WPM (George et al. 2008). This could be toxic for *A. radiata*, producing shoots damage. The presence of excessive amount of ammonium on MS medium has been also reported how one of the causes of tip necrosis (Gomes et al. 2014). While WPM medium has 4.99 meq L^{-1} of NH_4, MS has 20.61 meq L^{-1} (George et al. 2008).

In general, basal mediums to woody plants propagation have a higher concentration of sulfur, magnesium, iron, and manganese. WPM contributes higher amounts of sulfate that MS medium, 14.36 versus 3.0 meq L^{-1} (George et al. 2008), that could be other explication of the best result shown by the microplants cultivated in WPM basal medium.

About DKW medium, this didn't show a better effect than the control. As it has been mentioned, the calcium deficit is one of the most common causes of STN during plant tissue

culture (Bairu et al. 2009). For that reason, the use of the DKW medium, which provides higher amounts of calcium than MS basal medium, it could have been a good alternative to reduce the necrotic damage. Despite this, the incidence of STN wasn't significantly different between both basal mediums.

Plants height was affected by DKW in a similar way that calcium supplementation (0.6 and 0.9 g L^{-1} of $Ca(NO_3)_2$). Probably, because of the extra calcium contributed by DKW medium come from calcium nitrate. These treatments increased the height of the vitroplants. This way, calcium could be the answer. This component doesn't only have a structural role on cell walls, it also plays a performant on cell transport and signaling (Biaru et al. 2011). An increasing of calcium in the growth medium could affect minerals, hormones and vitamins movement, producing taller plants.

About fresh weight, dry weight and water content (%), no significant differences were observed between the control and the treatments. It could be that the number of measured plants during the trials (n=10) was not enough to detect the effects of the medium changes over de biomass accumulation of the microplants.

Conclusion

The use of WPM basal medium reduced significantly STN during *A. radiata* micropropagation, increasing multiplication rate of the microplants.

IBA supplementation also reduced STN of *A. radiata* and 0.01 mg L^{-1} of IBA improved the multiplication rate as well. The highest concentrations, as 0.05 and 0.1 mg L^{-1} of IBA, reduced multiplication rate. Probably, because of the induction of apical dominance, which decreases the regeneration of lateral shoots.

Agar treatments didn´t produce significant effects over the microplants. Despite this, 8 g L^{-1} of agar slightly reduced STN. Thus, it is a good option as agar concentration for *A. radiata* micropropagation.

The supplementation with calcium nitrate showed the worst response, generating large level of STN. It is probably that *A. radiata* microplants are more sensitive to nitrate which could be producing a toxic effect over them. On the other hand, apparently more than calcium deficit, other components of the basal medium are playing a more relevant role in the development of STN.

This way, the proposed medium recipe for *A. radiata* micropropagation is WPM basal medium, including its vitamins, supplemented with 0.01 mg L^{-1} of IBA, 0.5 mg L^{-1} of BAP, 8 g L^{-1} of agar, 30 g L-1 of sucrose, and pH 5.8.

Although WPM basal medium and IBA fixed the damage of the plants, STN is the result of a complex set of different factors which change between species. It is necessary make more research to determinate specific causes and the physiological processes associated to them.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Chapter 4: General Discussion

Domestication and Breeding of Native Chilean Plants and the role of Biotechnological Tools

Besides the general and specifics objectives proposed during the genesis of this research, the main goal of the investigation was to generate new knowledge about the domestication and breeding of Chilean native plants, in our case, *Argylia radiata*. With this experience, we hope to promote and to encourage the use of our genetic resources, generating new opportunities for our farmers, producers, and researchers.

Ornamental crop industry is characterized by wide range of products (cut flowers, potted plants, foliage, bushes, trees, flowering leaves, etc.) and by rapid changes in the varieties (Beruto 2013). This demand for new and innovative crops has made that The Netherland be the ornamentals world leader, with around 13% of the global market (Aurora IERUGAN, 2010). This Holland leadership is the result of the joint work between public and private entities, who with governmental support, have achieved to generate an innovative model of production and varieties development (Van Tuyl 2012). Similar situation is observed on USA and Japan. On the other hands, in the last years, European countries have been lost ornamental crops surface, while third world countries have been increased their production (Altmann 2016). This is caused because the European companies move their productions, including varieties, to places where labors are cheaper. Africa and Latin America are good examples of that. Colombia and Ecuador have become important producers and exporters of cut flowers, while Chile is the first South American producers of ornamentals bulbs (Cortez 2014). Around 80 to 90% of the Chilean bulb production is exported (ODEPA 2019). Despite this, none of these countries are varieties producers, the plant material that they use in their productions are generated by companies from Europe, North America and Japan and they are who control the plant genetics.

In the case of Chile, ornamental crops are less developed. The size of the internal Chilean market, our dependence of foreign companies to obtain new varieties, and the little interest of the private companies to develop new technologies limit the growth of the industry.

Nonetheless, the potential that our country has is enormous, especially because of our germplasm resources. Chile has several species, from many different ecosystems, with huge ornamental potential. That opens the opportunity to develop novel crops adapted for varied cultivation conditions. On the other hand, Chile has many genetic resources of relevant ornamentals. One example is *Alstroemeria*. Chile is an origin center for this crop, with around 45 different species (Muñoz-Schick, 2003), each of them with multiple subspecies and forms, transforming in a huge source of new genes. Similar are the cases of *Calceolaria* with 50 species, (Ehrhart, 2000), *Berberis* 16 species (Gómez et al., 2008), and *Gaultheria* with 12 species (Teillier and Escobar, 2013). Finally, Chile also has many no cultivated species, from families with huge ornamental potential such as *Amaryllidaceae*, *Asteraceae*, *Bromeliaceae*, *Cactaceae*, and *Solanaceae*. *Argylia radiata* is one of this resources, but the limited study of this kind of plants hinders its use. That way, investigations that put the focus native resources could help to develop new business opportunities, not only for the ornamental sector but also for other forest and agricultural industries.

Chilean researchers have had some experiences in native plants domestication and breeding. *Nothofagus* species, for timber proposes, have been cultivated and selected by several public institutions and private companies from 1976 (Castillo and Moreno, 2000), but still, *Pinnus radiata*, Douglas fir (*Pseudotsuga menziesii*), and different species of eucalyptus dominate the market. INIA has a long history with murtilla (*Ugni molinae*) breeding program, achieving to develop two varieties Red Pearl and South Pearl. New selections of murtilla are being evaluated and some others native berries, as Calafate (*Berberis microphylla*), are starting their domestication and study. Maqui berry (*Aristotelia chilensis*) is other example. This fruit has the highest concentration of antioxidants of all known fruits. This triggered a huge interest in this species, putting in risk the wild populations of maqui. This way, Talca University began a project of domestication and breeding in 2007,

developing advanced selections with better agronomic characteristics (Redciencia 2017). Now it is possible to find maqui orchards, products as energy drinks, lyophilized supplements, and candies, but still the production and commercialization of these native berries are marginal compared with conventional berries as blueberry, strawberries, and raspberries.

About ornamentals, it has been some experiences such as: *Chloraea* orchid species (Verdugo and Silva 2006; Verdugo et al. 2013); *Alstroemerias* (Olate et al. 2007; Nuñez et al. 2013; Aguirre et al. 2017; Aros et al. 2017; Gebauer et al. 2017); Copihue (Chait et al. 2013); native bushes (Vío et al. 2011); grasses (Schiappacasse et al. 2013; Nazal and Acuña, 2013); and Ornamental trees (Romero-Mieres et al. 2013). Probably, the most remarkable case is the breeding program of "Huillies" *Leucorynes* species, not only because the time that they have been worked also because this program produced three protected varieties: Paulina, Elena, and Gabriela. Nevertheless, none of these projects have could obtain yet commercial products with a huge impact on their industries. The above, probably it is the results that there has not been a joint work between investigation centers, universities, researcher, producers, and purchaser, which is extremely necessary to get products that have a relevant impact over the market (Beruto 2013).

The use of biotechnological tools is essential for plant domestication and breeding, facilitating the obtaining of new crops. In this sense, micropropagation and polyploid induction have been played a relevant role during the development of different horticultural crops (Rahman and Paterson, 2010; Bradshaw, 2016). The systematic use of polyploidization could bring many new options for the domestication of native Chilean plants, not only for potential ornamentals, but also for other kinds of crops, and the development of effective protocols of tissue culture helps to propagate the outstanding plant material, accelerating the development of new crops from native germplasm.

In the case of *Argylia radiata*, our research achieved to get valuable information about its biology, as morpho-anatomical and physiological adaptations, which allow to live in the driest desert of the world. This basic information, not only gives some lights about the evolutionary process of *A. radiata*, it could also help to establish cultivate programs, which support the *ex situ* production of this species.

About micropropagation, an effective protocol was developed. On our research, tissue culture was the base of the work, because allowed to get enough plant material for all the experiments, it made possible to generate a small germplasm bank, it facilitated the interchanging of plant material between countries, and eased the polyploidization process. At the same time, a good micropropagation system makes available the multiplication of outstanding individuals, helping with the development of new varieties in the futures programs of breeding. Despite this, it is necessary to follow with the investigation because of the hardening protocol is not well adjusted yet.

Regards polyploidization, the induction over in vitro plants with oryzalin allowed the obtaining of one mixaploid line 2n/4n (75/25% respectably). Although it was not possible to generate pure tetraploid material, this is a first step. This plants could be used to produce a complete tetraploid by buds isolation, to obtain polyploid pollen, and to be part of future breeding programs.

The direct use of this type of biotechnological tools, as micropropagation and polyploidization, over native species, was the bet of our research because their use could support in an efficient way the generation of novel forms of to utilize our plant genetic resources, impacting the agricultural worldwide industry.

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Chapter 5: Conclusions

Conclusion by chapter and general conclusion.

This doctoral research search to stimulate the sustainable use of our plant genetic resources through micropropagation and polyploidization of *Argylia radiata*. This understudied species was selected for this investigation because of the huge ornamental potential. The limited knowledge about this plant forced us to organize and to unveil basic biological information as a first step.

After that, the micropropagation was researched to develop an *in vitro* support for this and future projects. Micropropagation is a powerful tool to work with plants as *A. radiata*, not only because it allows rapid propagation of outstanding materials, but also because, micropropagation makes available plant materials which are difficult to obtain or depend on a random phenomenon as “Flowering Desert”.

Finally, polyploid induction trials were run, obtaining mixaploid diploid/tetraploid material, which can be used in futures projects.

Conclusion by Chapter

Chapter 2: Morpho-Anatomical adaptations of Argylia radiata (L.) D. Don to an arid environment

A. radiata presences morpho-anatomical adaptations that favor photosynthetic processes over water losses. This makes to think that the massive tuberous roots of *A. radiata* has an important reserve function. But our analysis, Lugol test included, showed that not starch is stored in the root tissue and its anatomical structure is similar to aquifer parenchyma of cactus, which acts as water store. Probably, those adaptations promote photosynthesis but do not store carbohydrates. One possibility is that the photoassimilates which are being stored are not Lugol reactive or the studied material has not enough reserves to detecting them. Another one is that those photoassimilates are used to generate biomass and to

complete the reproductive cycle during the short time in which Atacama Desert can support plant growth. It is necessary to further study the types of photoassimilates that are not Lugol reactive, that *A. radiata* produces and stores. Moreover, the morpho-anatomical characteristics of *A. radiata* that allow it to grow in desert areas make this plant a good candidate for development as an ornamental plant for use in xeriscaping.

Chapter 3: Control of shoot tip necrosis during *Argylia radiata* (L.) D. Don micropropagation

Previous trials showed that the micropropagation of *A. radiata* is possible even on free hormone MS medium (see appendix 1), but the addition of cytokinins, specially BAP, increases the multiplications rate in a significative way. Despite this, the use of those kinds of hormones generates tip shoot necrosis over the vitroplants.

The use WPM medium as basal medium and IBA supplementation reduce the incidence of the necrotic damage, improving the quality of the microplants and the multiplication rates of *A. radiata.*

***Argylia radiata* polyploidization**

Two inductors were tested over in vitro plants to generate polyploid material. Nitrous oxide was not effective to obtain polyploids, while the use of oryzalin achieved to develop one mixaploid 2n/4n, which could be used in futures breeding programs.

The used methodology with *A. radiata* (micropropagation and polyploidization) can be also use over other native plants to work with them.

General conclusion

Argylia radiata micropropagation and polyploidization were possible and in the frame of this project an effective protocol of micropropagation was developed, but the work with the

hardening process has to be following because it is not completely solved. About polyploidization, one mixaploid line was generated. This material could be used in the future breeding programs of this species.

Appendix 1: *Argylia radiata* Micropropagation, a Biotechnological Tool to Domesticate a New Ornamental Crop

Work presented, through oral presentation, at the International Symposium on Wild Flowers and Native Ornamental Plants. International Society of Horticultural Science (ISHS), May 1-4, 2017, Ramsar, Iran. Full text accepted to publication on respective Acta Book.

Argylia radiata Micropropagation, a Biotechnological Tool to Domesticate a New Ornamental Crop

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Abstract

***Argylia radiata* (L.) D. Don (Fam.: *Bignoniaceae*) is a perennial plant, native to the North of Chile, considered as a potential candidate for ornamental crop. The micropropagation was evaluated as part of the domestication process. Multiplication trial was run using MS medium supplemented with three types of cytokinins: BAP, kinetin and zeatin, with 4 different concentrations (0.5, 1.0, 1.5 and 2.0 mg L^{-1}) and free hormone medium as control media. After 5 weeks, the number of sprouts, multiplication rate, plant height, and plant damage were measured. Fresh weight, dry weight and water content of the plants were determined as well. Medium containing 1.0 mg L^{-1} of BAP produced the biggest shoots emission (8,45 sprouts per plant). The highest multiplication rate (7,22 explants per plant) was observed in the 2.0 mg L^{-1} of BAP and the lowest was observed in kinetin media. Zeatin supplemented media showed an increase in the damaged plants but no damage was observed in the plants cultured in hormone free medium. Plants in control medium and kinetin supplemented media showed the lowest fresh and dry weights while those in media containing BAP 2.0 mg L^{-1} had the highest. The plantlets in hormone free medium had the lowest water content as well. Finally, the best multiplication rate was obtained in the media supplemented with BAP and zeatin.**

Keywords: *Argylia*, *Bignoniaceae*, ornamental, micropropagation, Chilean native plant.

INTRODUCTION

Argylia radiata (L.) D. Don is a perennial plant, belonging to *Bignoniaceae* family and native to arid areas in the North of Chile. It is part of the "Blooming Desert" and grows in salty soils, under extremely dry conditions (Riedemann et al., 2016). This species can be a new option for ornamental crop because of its green-blue foliage and multiple floral stems with beautiful trumpet flowers. Flower color ranges from light yellow to deep red with tones of orange and pink. The extremely hard conditions in which these plants grow could make them a candidate for landscaping due to their low water requirement. As part of the domestications process of *A. radiata*, a protocol of micropropagation was tested.

MATERIALS AND METHODS

During 2015, herbaceous shoots of *Argylia radiata* were collected from Bahía Inglesa, North of Chile. They were dipped in a fungicide solution (Captan 20 g L^{-1}) for 30 minutes, then passed through an ethanol solution at 70% (v/v) and they were washed in a solution of commercial bleach at 20% (v/v) for 20 minutes. After that, the material was rinsed three times, each of them for 5 minutes, with sterile water. The explants were cut eliminating all damaged

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tissue. Finally, nodal sections were planted in MS medium supplemented with 30 g L^{-1} of sucrose, 6.5 g L^{-1} of agar, at pH 5.8. After 8 weeks, the new sprouts were isolated to fresh medium.

Multiplication trial was run using complete MS medium with three kinds of cytokinins including BAP, kinetin and zeatin, at 4 different concentrations (0.5, 1.0, 1.5 and 2.0 mg L-1), as well as a MS medium without hormones as control medium. The media were supplemented with 30 g L^{-1} of sucrose and 6.5, g L^{-1} of agar. The pH was adjusted to 5.8. 65 plants composed of 3-4 nodes were established under laminar flow chamber. After 5 weeks in growth room (23°C ± 2°C, 40% of humidity and 16 light hours), the number of sprouts, multiplication rate, plant height and plant damage were measured for 40 plants. To determine the damage level, we defined a scale from 0 to 5: 0=no damage, plants without necrosis; 1=soft damage, less of 25% of the tissue shows damage; 2=medium damage, 25% to 50% of the plant's tissue shows necrosis; 3=severe damage, 50% to 75% of the tissue has necrosis; 4=serious damage, over 75% of the plant's tissue shows necrosis; 5=dead plants, it is not possible to obtain new explants. Fresh and dry weigh of 25 plants were measured. The dry weight was obtained after dehydrating the plants in an oven for 48 hours at 70°C. The water content (%) was calculated as the difference between fresh and dry weight. All the data were analyzed using Kruskal-Wallis test.

RESULTS AND DISCUSSION

Table 1 shows the multiplication parameters of argylia microplants, while in table 2, we can see the *in vitro* growth parameters of each media.

Table 1. Multiplication parameters of *in vitro* plants of *Argylia radiata* cultivated on different growth media.

Medium	n	*In vitro* multiplication parameters			
		Number of sprouts (sprouts per plant)	Multiplication rate (explants per plant)	Plant height (cm)	Damage level[1]
Free hormone medium	40	2,48^{cd}	2,50^{de}	4,74^{abc}	0,05^{a}
BAP 0,5 mg L^{-1}	40	6,28^{b}	3,60^{cd}	4,17^{bcd}	1,20^{efg}
BAP 1,0 mg L^{-1}	40	8,45^{a}	3,68^{bc}	4,18^{cd}	0,80^{def}
BAP 1,5 mg L^{-1}	40	8,03^{ab}	3,78^{bc}	3,46^{e}	1,03^{def}
BAP 2,0 mg L^{-1}	36	6,58^{ab}	7,22^{a}	3,58^{e}	0,69^{cde}
KIN 0,5 mg L^{-1}	40	2,13^{d}	1,78^{ef}	3,76^{de}	0,43^{abc}
KIN 1,0 mg L^{-1}	40	2,58^{cd}	2,18^{ef}	4,36^{bcd}	0,35^{ab}
KIN 1,5 mg L^{-1}	39	2,08^{d}	1,65^{f}	4,23^{bcd}	0,53^{bcd}
KIN 2,0 mg L^{-1}	40	3,53^{c}	2,23^{ef}	4,55^{abc}	1,18^{ef}

ZEA 0,5 mg L^{-1}	40	5,80^{b}	3,33^{cd}	4,97^{a}	1,60^{gh}
ZEA 1,0 mg L^{-1}	39	6,90^{ab}	4,33^{bc}	4,74^{ab}	2,08^{h}
ZEA 1,5 mg L^{-1}	40	5,65^{b}	4,33^{b}	4,76^{ab}	1,43^{fg}
ZEA 2,0 mg L^{-1}	40	6,58^{ab}	4,73^{b}	4,47^{abc}	1,73^{gh}

Different letters indicate significant differences between groups ($p<0,05$).
[1]Numeric scale from 0 to 5, where 0=no damage and 5=dead plants, it is not possible to obtain new explants.

Medium containing 1.0 mg L^{-1} BAP showed the highest shoot proliferation (8,45 sprouts per plant), but it was not significantly different from media supplemented with 1.5 mg L^{-1} BAP, 2.0 mg L^{-1} BAP, 1.0 mg L^{-1} ZEA, and 2.0 mg L^{-1} ZEA. No significant differences were observed between free hormone medium and kinetin media.

The largest multiplication rate (7.22) was observed in medium containing 2.0 mg L^{-1} BAP and the lowest was shown in kinetin media. The free hormone medium showed a multiplication rate of 2.50, which proves that the addition of cytokinins, especially BAP and zeatin, improves the multiplication rate of argylia. The increase of multiplication rate by cytokinins has been reported in several species (Ozden-Tokatli et al., 2005; Panicker et al., 2007; Arab et al., 2014).

Zeatin media and free hormone medium showed the longest shoots, and no significant differences were found between them. The smallest plants were observed in the BAP media. Here, we could see a trend; the plant height was reduced by the increase of hormones, BAP and zeatin. Kinetin media showed a different situation; we observed an increment of plants height with the increase of the hormone concentration. Furthermore, plants in medium containing 0.5 mg L^{-1} KIN were significantly smaller than those in medium with 2.0 mg L^{-1} KIN.

Zeatin media showed an increase in plant damage, but values of damages remained in acceptable level. We observed no damages in the plants grown in free hormone medium. This damage can be caused by multiple factors like mineral deficiencies, wrong pH, excesses of temperature and light, or even the growth promoting effect of cytokinins that could causes nutritional deficiencies during the plants growth. That can explain why the plants grown in free hormone medium showed the softest damage. The change in the mineral base or the addition of calcium have been described as effective ways to reduce the damage of the plants (Tetsumura et al., 2008; Srivastava and Joshi, 2013; Amalia et al., 2014).

Table 2. Growth parameter of *in vitro* plants of *Argylia radiata* cultivated on different growth media.

Medium	n	*In vitro* growth parameters		
		Fresh weight (mg)	Dry weight (mg)	Water content (%)
Free hormone medium	25	88,64^{i}	15,91^{h}	81,14^{a}
BAP 0,5 mg L^{-1}	25	385,62^{cd}	35,02^{abc}	90,44^{de}

BAP 1,0 mg L^{-1}	25	583,09^{abc}	44,51^{ab}	91,42^{fg}
BAP 1,5 mg L^{-1}	25	596,23^{ab}	39,16^{abc}	93,16^{h}
BAP 2,0 mg L^{-1}	24	664,35^{a}	47,53^{a}	92,54^{gh}
KIN 0,5 mg L^{-1}	25	142,56^{hi}	18,82^{fgh}	85,84^{ab}
KIN 1,0 mg L^{-1}	25	163,13^{hi}	17,09^{gh}	88,67^{cd}
KIN 1,5 mg L^{-1}	17	196,81^{ghi}	21,46^{fgh}	87,89^{bc}
KIN 2,0 mg L^{-1}	25	223,81^{fgh}	22,28^{efg}	89,27^{cd}
ZEA 0,5 mg L^{-1}	25	242,98^{efg}	26,37^{def}	88,16^{de}
ZEA 1,0 mg L^{-1}	25	373,39^{bcd}	34,27^{abc}	90,45^{ef}
ZEA 1,5 mg L^{-1}	24	297,76^{def}	29,72^{cde}	90,04^{de}
ZEA 2,0 mg L^{-1}	24	336,75^{de}	33,61^{abc}	89,58^{cde}

Different letters indicate significant differences between groups ($p<0.05$).

For the three analyzed cytokinins, the fresh and dry weights raised with increase in hormone concentration. The water content increased with increase in hormone concentration as well. Free hormone medium and kinetin media showed the lowest fresh and dry weights, while media containing BAP 2.0 mg L^{-1} had the highest accumulation of biomass (fresh and dry weights). This was probably caused by cell division effect of cytokinins.

Free hormone medium had the least water content (81.14%). This can help to the acclimatization of the microplants because the reduction in the water content on the plant tissue reduces the dehydration risk when taken out from the laboratory to the greenhouse. On the other hand, the increase in the water content of the plants may be a sign of hyperhydricity problem. This reduces the quality of the plants, complicating the micropropagation and hardening processes (Gao et al., 2017).

CONCLUSIONS

The micropropagation of *Argylia radiata*is is possible using MS basal medium and sucrose supplementation, even in a free hormone medium.

The addition of BAP and zeatin increased the multiplication rates of *A. radiata* on *in vitro* culture. Kinetin was found to have no effects on multiplication rate and number of shoots.

BAP and zeatin media caused certain level of damage to the microplants, which makes it necessary to continue with more essays to improve the plants condition.

An increase in water content was observed at higher hormone concentration. The increment of hormone concentration should be carefully done as vitrification problems may occur.

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Appendix 2: Polyphenolic distribution in organs of *Argylia radiata,* an extremophile plant from Chilean Atacama Desert.

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Polyphenolic distribution in organs of *Argylia radiata*, an extremophile plant from Chilean Atacama Desert

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The polyphenolic distribution on different organs of *Argylia radiata,* an extremophile plant from the Atacama "Flowering Desert", was studied. The total polyphenolic and antioxidant capacity of ethanolic extracts from leaves, tuberous root and flowers of different colors were evaluated. Orange and red flowers showed the highest polyphenolic and flavonoid content. The maximum anthocyanin concentration was found in red flowers and the antioxidant activity (ABTS and FRAP) of extracts changed according to the organ. The HPLC-MS/MS analysis of the extracts allowed to identify 10 new polyphenols belonging to different families. Rutin was identified as the most abundant polyphenol in all plant organs, followed by quercetin and coumaric acid. The distribution and composition of polyphenols on *A. radiata* organs are presented herein for the first time. Their role in plant response to abiotic and biotic stress, their use for chemotaxonomic purposes as well as their potential biotechnological application are discussed.

Keywords: extremophile plant • polyphenols • antioxidant • flavonoids • HPLC-MS/MS

Introduction

An extremophile is a terminology used to refer to organisms which live in harsh environments as arid climate. In the case of plants, this concept could cover a wide range such as ephemerals, geophytes, perennials, bushes and trees, all of them with different adaptations to survive on hard ecosystems [1]. Argylia radiata (L.) D. Don is an herbaceous perennial plant belonging to the Bignoniaceae family, Tecomeae tribe [2]. This plant is native from one of the driest areas in the world, that includes northern Chile (from Arica and Parinacota to Coquimbo Region) and southern Peru. Argylia is a representative species of the "Atacama Flowering Desert", a sporadic event climatically associated with "El Niño" phenomenon, which provokes unusually high rainfalls in this area. Most plants of the Bignoniaceae family inhabit tropical and subtropical regions [3]. As an exception of this family, A. radiata has developed some physiological and morpho-anatomical adaptations to survive in the world´s driest desert. The most striking one is its tuberous root, which can reach a significant weight and several centimeters of length. Taking into account these facts, A. radiata could become a valuable research tool for a better understanding of the plant's adaptation mechanisms to survive in desert zones under harsh conditions.

It is known that most plant species that live under extreme environments, usually have an active secondary metabolism (phytochemicals), giving those plants interesting properties from the biotechnological point of view. In desert areas, plants growth under concomitant abiotic stresses such as salty soil, high temperature, drought, and high UV radiation at the same time [1]. In this regard, different surviving strategies have been adopted by plants in nature. For example, halophytic flora has developed tolerance to salinity stress, with various adaptive mechanisms such as ion compartmentation or ion transport and regulation [4]. High temperature and high UV radiation could cause plant oxidative stress, provoking highly reactive oxygen

species (ROS) formation. Several pathways for ROS scavenging have been described in extremophile flora, including the expression of antioxidant enzymes, osmoprotectant compounds production (e.g. betaine, polyols) or the action of antioxidant compounds like polyphenols [5][6]. Recently, the interest in naturally produced antioxidant polyphenolic compounds has increased, due to its potential applications as an antibiotic replacement or as food biopreservative [7]. For example, the antioxidant properties of polyphenolic compounds from Muhaisnah desert extremophile plants, such as *Fagonia indica*, *Zygophyllum hamiense*, *Salsola imbricata*, and *Calotropis procera* had been studied [8]. By another side, strong antioxidant properties and inhibition of lipid oxidation activity have been observed in plants from semiarid regions of Mexico (*Jatropha dioica*, *Flourensia cernua*, *Eucalyptus camalduelnsis*, and *Turnera diffusa*) [9]. Moreover, the edible halophyte *Suaeda fruticosa*, from Tunisian arid regions, has been studied because of its anti-inflammatory activity and as a potential source of antioxidants [10].

Previous phytochemical studies performed on *A. radiata* leaves and roots extract found thirteen compounds belonging to the family of iridoids. It can be mentioned the presence of argylioside; catalpol; plantarenaloside; 8-epi-7-deoxyloganic acid; 7-deoxygardoside [11]; radiatoside [12]; geniposidic acid; mussaenosidic acid; radiatoside B and C [13]; radiatoside D [14]; radiatoside E, and radiatoside F [15]. However, to the best of our knowledge, the *Argylia* polyphenolic composition and their distribution in the plant organs as well as their antioxidant capacity, have not been studied yet.

The purpose of this work was to study the polyphenolic composition and the antioxidant capacity of *A. radiata* ethanolic extracts from different plant organs. For that purpose, total polyphenolic content, total flavonoid content, total anthocyanin content, as well as the antioxidant capacity (ABTS and FRAP), were measured in plant leaves, root, and flowers from different colors. Furthermore, relevant polyphenolic compounds from different families were identified and quantified HPLC-MS/MS. Those results will allow scientist to understand the secondary metabolism of this species and determining if *A. radiata* could be also used as a source of bioactive compounds for future biotechnological applications.

Results and Discussion

Plant characteristics and collection site

A. radiata is a typical plant from Chilean “Flowering Desert”, which rapidly sprout when winter rainfall is sufficient. As a hemicryptophyte plant, the shoots, leaves, and flowers are discarded to survive during the harsh drought period. However, this species keeps the tuberous storage roots, which is one of the most remarkable characteristics of them. Roots are located from 10 to 30 cm under soil profile, bearing the renewal buds in their upper part. They can reach an important size, up to 70 cm long, and weight near to 3 Kg, which means these roots are used to store water and photo-assimilates. By other side, winter rains in the Atacama Desert are a random phenomenon, associated with ENSO event (El Niño/Southern Oscillation), that could take several years before manifesting [16]. This fact could limit the accessibility to the *Argylia* plant material, because of their aerial parts are available only during these events period. The latter could be one of the reasons why research on this extremophile plant has been scarce so far. In figure 1 the area where the samples were collected is shown and the climatological data of this zone is presented.

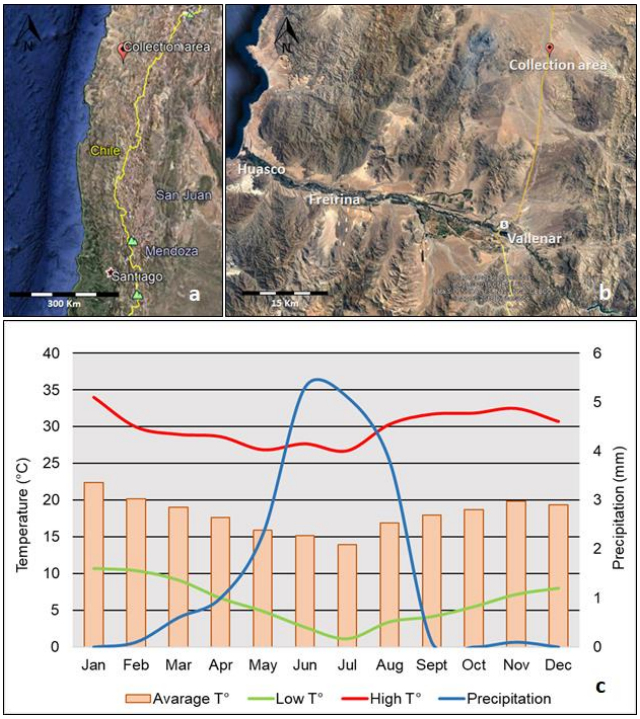

Figure 1. Geoclimatic information about the area where *Argylia* samples were collected. (a) Location of the collection area. (b) Close up of the collection area near to Vallenar city at Atacama Region. (c) Climatological information of the area. The chart was made using the average of the last 10 years of temperatures and precipitations. All data were obtained from the Agromet INIA database [18].

The monthly average temperature from this area range from 14 to 22°C, with a maximum of 34°C in January and a minimum of 2°C in July. It could be observed that is some areas, the annual precipitations are less than 12 mm, which is in agrement with previous reports [17]. Besides, larger dryness period of 9 months, with precipitations lower than 1 mm, reveals the extreme conditions of the Atacama desert, which is the natural habitat of *A. radiata*. Moreover, soil analysis of collection sites shows basic pH (7.35 to 7.95) and high concentration of nitrogen (22.7 to 110 ppm), potassium (84.5 to 533 ppm), calcium (744 to 3422 ppm), magnesium (75.4 to 352 ppm), and copper (0.50 to 3.06 ppm). All of them are essential nutrients, but at high concentrations, they could cause phytotoxicity.

Phytochemical characterization

For *A. radiata* phytochemical characterization, the samples were divided by plant organs, this means plant leaves (LV); root cork (RC), and root pulp (RP) (Figure 1). Regarding flower samples, they were grouped by colors having flower ranging from yellow (YL1, YL2), orange (OR1, OR2) to red ones (RD1, RD2, RD3, RD4, RD5). The polyphenolic composition and the antioxidant capacity of all extracts are summarized in table 1.

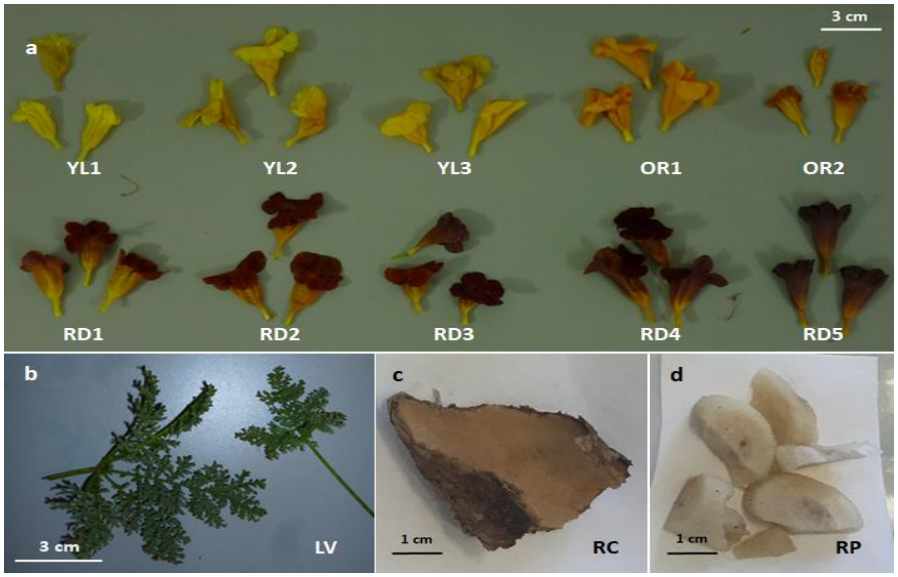

Figure 2. Types and codes of *A. radiata* samples. (a) Flowers were separated by color, from lightest ones to darkest: YL=yellow, OR=orange, RD=red. (b) Leaves (LV). (c) Tuberous root cork (RC). (d) Tuberous root pulp (RP).

Table 1. Total polyphenolic content, flavonoid content, total anthocyanin and antioxidant capacity determined in extracts from *A. radiata* samples.

Sample	TPC (mg GAE[a]/g FW)	Total flavonoids (mg QE[b]/100g FW)	Total anthocyanins (mg eq CG[c]/100g FW)	ABTS$^{\cdot+}$ (mmol Trolox/g FW)	FRAP (µmol FeSO4/g FW)
YL-1	5.6 ± 0.1	39.4 ± 0.2	2.0 ± 0.13	0,76 ± 0.01	0,6 ± 0.02
YL-2	2.5 ± 0.1	55.5 ± 0.2	2.0 ± 0.08	0,46 ± 0.01	0,4 ± 0.01
YL-3	5.5 ± 0.2	122.3 ± 0.2	8.9 ± 0.21	0,65 ± 0.01	1,1 ± 0.03
OR-1	14.7 ± 0.2	103.5 ± 0.7	1.9 ± 0.13	0,39 ± 0.01	0,9 ± 0.02
OR-2	4.6 ± 0.2	43.5 ± 0.2	2.1 ± 0.17	0,76 ± 0.01	0,8 ± 0.02
RD-1	6.1 ± 0.2	114.7 ± 0.9	107.9 ± 2.5	0,26 ± 0.003	1,2 ± 0.02
RD-2	6.9 ± 0.2	55.7 ± 0.1	146.2 ± 2.7	0,83 ± 0.01	1,2 ± 0.01
RD-3	8.2 ± 0.1	109.8 ± 0.2	340.2 ± 3.1	0,35 ± 0.002	1,4 ± 0.01
RD-4	12.2 ± 0.2	180.1 ± 0.1	239.9 ± 2.0	0,47 ± 0.01	2,0± 0.02
RD-5	14.8 ± 0.2	187.6 ± 0.2	568.1 ± 1.9	0,54 ± 0.01	2,4± 0.02
LV	15.2 ± 0.3	126.6 ± 0.2	--	0,39 ± 0.01	3,0± 0.02
RC	0.7 ± 0.1	5.7 ± 0.1	--	0,41 ± 0.01	0,2± 0.02
RP	ND	ND[d]	--	0,46 ± 0.01	ND[d]

[a]GAE = Gallic Acid Equivalents; [b]QE=Quercetin equivalent; [c]CG = cianidine-3-glucoside; [d]ND= No detected; TPC = Total polyphenolic content

Different conditions such as high temperatures, salinity, UV radiation, drought, oxygen deficiency, freezing, and heavy metals can induce stress on plants. In this sense, polyphenols play an important role in the stress response. Facing a stressful condition, the formation of reactive oxygen species (ROS) is induced [19][20]. To avoid their harmful effects on cells the plants have developed two response pathways, the enzymatic and the non-enzymatic. In the case of the enzymatic pathway, it is mediated by enzymes that degrade ROS, meanwhile, in the non-enzymatic pathway, the polyphenols act as antioxidant components [21]. Furthermore, polyphenols can interact directly with other cellular components, regulating cell processes as mitosis, cell elongation, senescence, and cellular die [22]. They also can chelate metal ions [21] and protect crops against insect pest [23], playing an important role in plant development under stress conditions. The presence of polyphenols in plants

is determined by both genetic and environmental factors [24]. In this sense, it seems that a desert plant, as *A. radiata*, accumulates a high polyphenolic concentration in leaves as one of their protection mechanism from abiotic stress factors.

Although no specific trend has been established regarding secondary metabolites accumulation in different plant organs, it could be observed the highest polyphenolic concentration in *A. radiata* leaves (15.2 mg GAE/g FW), followed by flowers and roots (0.7 mg GAE/g FW), respectively. The phenolic content differences found among plant leaves and roots had been previously observed for other plants [25]. This may be due to plant aerial part is unprotected to biotic and abiotic stress conditions. In fact, the aerial parts of the plants are more exposed to abrupt environmental changes (temperature, wind, UV radiation), and plagues attack than roots. Then, high polyphenolic production could be one of their adaptative responses to survive in this habitat. Moreover, TPC in *A. radiata* leaves are 5 times higher than in *Calligonum comosum*, and 12 times higher than in *Calligonum azel*, respectively, both species are wild plants from Tunisia desert [26][27]. Moreover, this is also 41 times higher than TPC quantified in shoots of the halophyte medicinal-plant *Suaeda fruticosa*, a species that habitat in Mediterranean salt marshes [10] but 6 times lower than reported for *Baccharis tola* that growth in the Atacama Desert [28]. By another side, it could be observed that TPC increases from yellow flowers (YL) to the red ones (RD) falling their values in the range from 0.2 to 1.9 mg GAE/g FW (Table 1). In fact, OR-1, RD-4, and RD-5 showed higher TPC content among flowers being two or three times higher than for other flowers samples. Those TPC values were similar to those reported for *Malus* genus flower (4.4 to 18.4 mg GAE/g FW) [29] but lower than reported for *Pyrus pashia* (108.8 mg GAE/g extract) edible flower ethanolic extract [30]. These results suggest the potential application of Argylia flowers and leaves as a source of relevant polyphenols for using in food and biotechnology.

Anthocyanins are a family of compounds belonging to the general class of phenolic compounds known as flavonoids, which are the major flower pigments in higher plants [31]. Regarding both flavonoids and anthocyanin content, it is observed (Table 1) that their content changes from yellow to red flowers, different flower color would mean different product composition and/or concentrations. Flavonoid content showed a range from 0.2 -5.7 mg QE/g FW, being the red flowers samples RD-4 and RD-5 the one with higher content. Those values were 3 to 4-fold higher than YL-1, YL-2, OR-2, and RD-2 samples. *Argylia* leaves showed the third highest flavonoid content (1.3 mg QE/g FW) in the opposite to root cork sample where was found the lowest amount of these polyphenols. Similar content of flavonoid has been detected in another desert-growing plant such as *Calotropis procera* (1.2 mg QE/g extract) and *Fagonia indica* (2.20 mg QE/ g extract) but higher amount was found in *Suaeda fruticosa*, (26.2 mg QE/g DW), *Atriplex tatarica* (146.1 mg QE / g extract) and *Atriplex littoralis* (127.6 mg QE/g extract) [7][8][10].

The anthocyanin content showed by the red flower samples (1.1-5.7 mg eq CG/g FW) were 5 to 28 times greater than determined in yellow and orange flower samples (0.2-1.9 mg eq CG/g FW), respectively. It is important to highlight that values obtained for YL samples were very low. According to those results, it is possible to hypothesize that color of *Argylia* flowers is determined mainly by flavonoids in YL and OR varieties and by both flavonoids and anthocyanins accumulation in RD varieties. The flowers color expression is determined by the type of pigment present in the tissue and its intramolecular and intermolecular interaction with co-pigments, metals, etc. Usually, species with yellow and orange flowers are the product of the accumulation and combination of carotenoid, anthocyanins, chalcones, aurones and some flavonols, but other compounds such as flavones could act as co-pigments. Flavones, flavonols, chalcones, and aurones were successfully identified from yellow flowers of *Antirrhinum majus*, which is a member of a close family from *A. radiata* [31]. In the case of deep red, this color could be the result of the combination of anthocyanins, cyanidin, and carotenoids [32]. However, it should be necessary to make further analysis for identifying the exact pigments which determine the *A. radiata* flowers color. No anthocyanins were observed in leaves (LV) and tuberous root samples (RC and RP).

Antioxidant capacity

It is known that polyphenols may possess antioxidant activity that can be influenced by many factors, hence it is recommended to use various methods for a broader evaluation of plant material [33]. Therefore, the evaluation of the *Argylia* extract was carried out using two different antioxidant models: $ABTS^{\cdot+}$ and FRAP assays. $ABTS^{\cdot+}$ has been used extensively to assess the capacity of extracts to act as free radical scavengers. Flower extracts showed a very wide range of $ABTS^{\cdot+}$ values between 0.26-0.83 mmol Trolox/g FW, while leaf and root extracts showed values between 0.39-0.46 mmol Trolox/ g FW.

By another side, the FRAP assay was also used to assess the antioxidant activity of the samples. Tuberous root pulp (RP) showed no reducing power, while the tuberous root cork showed the lowest FRAP value of 0.2 µmol $FeSO_4$/g FW. For flower samples, a range between 0.4 and 2.4 µmol $FeSO_4$/g FW was observed, being the red flowers the ones with higher values. The leaf extract showed the highest value among all samples with 3.0 µmol $FeSO_4$/g FW. This antioxidant capacity could be due to the combination of all type of polyphenolic compounds found on each plant organ. It is known that the presence of secondary metabolites in plants that shown antioxidant capacity could help them to defend against both pathogens and stress conditions.

Higher antioxidant capacity was found in plant extract from semiarid Mexican region such as *Turnera diffusa* and *Flourensia cernua*, which presents ABTS radical inhibition ranging from 40 to 90% [9], and other halophyte species, especially from *Brassicaceae* family such as *Lepidium rudelare*, present lower antioxidant capacity when determining by a DPPH assay (IC_{50} 869,58 µg/ml) [7], while values of 10% where found for *Zygophylum hamiense* and *Salsola imbricata* DPPH radical scavenging inhibition activity [8].

HPLC-MS analysis

More the 8.000 polyphenols had been isolated from different plant organs [34], and their study has increased in recent years due to their multiple effects on human health. The anticancer capacity of the polyphenols through their antioxidant action, anti-inflammatory effect and their capacity to inhibit the proliferation and survival of cancer cells have been described [22]. Also have been reported: neuroprotective activity [35], protection against mutagenic substances [34], antifungal and antibacterial action [36], preventing osteoporosis [37], prevention of cardiovascular diseases [38], and positive effects over bones and joints [39]. Polyphenolic compounds from *A. radiata* flowers, leaves and roots were analyzed by HPLC-MS/MS and were identified and quantified as shown in Table 2. Moreover, phenolic classification by family and its respective abundance in plant organs are presented in figure 3.

Table 2. HPLC-MS/MS data of polyphenolic content from different *Argylia radiata* organs.

	Concentration (µg/g FW)												
	YL-1	YL-2	YL-3	OR-1	OR-2	RD-1	RD-2	RD-3	RD-4	RD-5	LV	RC	RP
Ferulic acid	2.2 ± 0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Chlorogenic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.9 ± 0.1	10.2 ± 0.1	0.3 ± 0.1	nd
Caffeic acid	nd	nd	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	nd	0.3 ± 0.1	0.2 ± 0.0	nd	0.5 ± 0.1	nd	nd
Coumaric acid	0.1 ± 0.00	nd	0.2 ± 0.0	0.1 ± 0.00	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	nd	nd	0.1 ± 0.0	nd	nd
Pinocembrin	nd	nd	nd	nd	nd	nd	nd	nd	0.1 ± 0.0	0.2 ± 0.0	nd	nd	nd
Rutin	22 ± 0.4	37.0 ± 0.3	63.6 ± 0.2	48.8 ± 0.4	45.0 ± 0.4	64.7 ± 0.5	68.6 ± 0.2	53.5 ± 0.2	63.2 ± 0.5	62.9 ± 0.3	28.3 ± 0.2	0.6 ± 0.1	0.1 ± 0.0
Quercetin	nd	nd	0.5 ± 0.1	nd	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	nd	nd	nd
Abscisic acid	nd	nd	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	nd	nd	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	nd	nd
Luteolin	nd	nd	nd	nd	nd	0.1 ± 0.0	nd	0.1 ± 0.0	nd	nd	nd	nd	nd
Vanillic acid	nd	nd	nd	1.6 ± 0.2	nd	nd	nd	nd	nd	nd	nd	nd	nd
Apigenin	nd	nd	nd	nd	nd	0.1 ± 0.0	nd	nd	nd	nd	0.1 ± 0.0	nd	nd

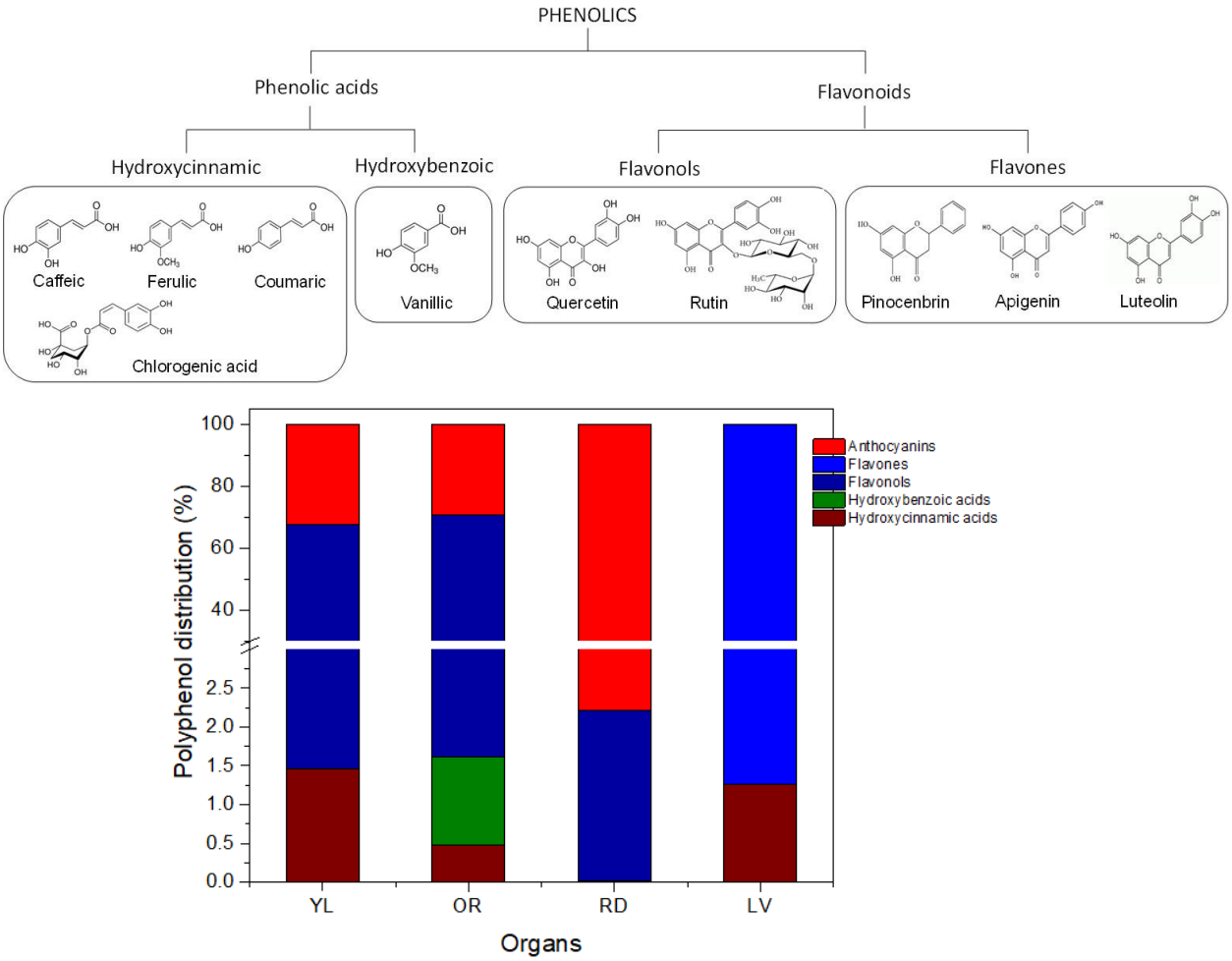

Figure 3. Classification and distribution of phenolic compounds found by HPLC-MS/MS analysis on *A. radiata* flowers, leaves, and root extracts.

Major compounds in plant organs were hydroxycinnamic acids and flavonols (figure 3). The most abundant phenolic found in all samples was rutin (Table 2), showing the highest concentration in flowers (22.0 to 68.6 µg/g FW), accounting for > 96 %. Whereas in leaves (28.3 µg/g FW) it represents near 73 % of quantified phenolic compounds. In *Argylia* root parts it was found in minor quantity (0.1 to 0.6 µg/g FW), regarding the other plant tissue, representing 75 % in root cork and 100 % in the pulp. This flavonol could contribute to color expression in YL and OR flower varieties and also to the antioxidant activity found in all plant organs, even in root pulp where only a negligible antioxidant activity was found. Rutin had been detected in the leaves of some species of *Tecomeae* tribe as *Jacaranda decurrens*, *Tabebuia caraiba* and *Tabebuia ochracea* [40]. It was also found in the medicinal plant from Muhaisnah desert *Zygophyllum hamiense*, accounting for 50% of the identified compounds and it represents 10% of the identified polyphenols in *Calotropis procera* and *Salsola imbricata* [8]. It was recently reported that rutin could have a potential defensive role against plant pest attack [23]. For instance, in resistant varieties of soya beans to *Piezodorus guildinii,* it was observed an increase of rutin synthesis after insect attack, decreasing pest proliferation, while in susceptible ones didn't show this increment. This findings suggesting this compound could play multiple roles in A. *radiata* stress response and adaptation to arid conditions. On the other side, pharmacological studies on rutin bioactivity reported anticancer and vasoprotective activity as well as protection against mutagenic substances [34].

Quercetin flavonoid was the second most abundant compound in most *Argylia* flower samples but at a lower concentration, around 0.5 µg/g FW. However, it was not detected on flower samples YL-1, YL-2, leaves, and roots. Such phenolic compound has been widely studied due to its broad distribution in plant leaves and fruit. It is known their antioxidant activity [34] and it has been commonly identified in plant extracts from other desert medicinal plants [8].

Chlorogenic acid is a well known phenolic compound that was identified in *Argylia* extracts, but only in one red flower variety, in LV and RC samples, respectively. In leaves it accounts for 38 % and in root cork for 25 %, respectively, playing an important role in the antioxidant properties determined in those tissues. This hydroxycinnamic acid is widely distributed in plant organs and its antioxidant activity had been previously reported [41], whereas biological tests on mice have shown its action against osteoporosis [37].

Caffeic acid was detected on some flowers samples and *Argylia* leaves, also have shown antioxidant activity [42]. This compound has been found in *Bignoniaceae* plants from different tribes [43]. Other polyphenols detected in lower concentrations were coumaric acid, which has been reported antibacterial and antifungal activity [44]. Lutein showed anti-cancer properties [45] and vanillic acid has been identified a protective effect on the liver [46], preventive action against gastrointestinal diseases [47], and activity on cardiovascular pathologies [48]. The coumaric and vanillic acid have also been isolated from several plants of *Bignoniaceae* family [43] while, luteolin was detected on plants from *Markhamia* genus, member of *Tecomeae* tribe [49]. Apigenin has an effect on the liver, prevents cardiovascular diseases, action on the respiratory system, positive effect on the endocrine system due to its activity on the pancreas, protection of nerve cells and activity on bones and joints [39]. Pinocembrin has the anti-inflammatory, antimicrobial, anticancer, antifungal and neuroprotective capacity [36]. Ferulic acid was detected only in YL-1 and it has antioxidant action, antiapoptotic activity [50], neuroprotective effects [35] and activity against heart disease [38]. In general, it was found that most compound identified in *Argylia* plant organs have been reported in other plants coming from tribes from the same family, and in closely related families as well [43]. For this reason, they could be used as phytochemical markers for this tribe. Furthermore, most of those secondary metabolites had been found in plants from desert habitat which would mean they are related to plant adaptation to those systems.

When comparing the relative abundance of the phenolic family quantified on different *Argylia* organs (Figure 3), a similar pattern can be observed for YL and OR samples, where flavonols are the main group detected. Whereas in YL and OR samples a small fraction of hydroxycinnamic acids were identified but hydroxybenzoic acids were only found in OR samples. RD samples showed that anthocyanins are the most abundant polyphenols followed by flavonols. Moreover, a difference in phenolic relative abundance was observed in the LV sample regarding flower ones, because there is no contribution of anthocyanins nor cinnamic acids, but flavones are major compounds. By other side, flavones were only detected in root samples where they were the most abundant phenolic family.

Finally, it is evident that *A. radiata* has huge biotechnological potential, probably encouraged by the environmental conditions in which it had evolved. However, it is necessary to continue doing research in this plant for exploring their domestication and production at large scale for both ornamental (indoor low water requirement plant) and industrial applications. *Argylia* plant will allow obtaining polyphenolic rich extracts with bioactive components relevant for food or biomedical applications, among others.

Conclusions

For the first time, ten new phenolic compounds with antioxidant properties were identified in Argylia radiata plant organs. Flowers and leaves contained a higher amount of such compounds on the opposite to roots. It seems that phenolic families distribution depends on plant organ type been flavonols and anthocyanins more abundant in flowers but flavonols in plant leaves. Several of the identified compounds may have plant anti-stress role, due to the antioxidant properties, allowing Argylia to survive under high temperature, high UV radiation and drought conditions from arid lands. All results suggested that A. radiate phytochemicals could be used for chemotaxonomic purposes and as active ingredients for other applications in food and cosmetic. Finally, this study could contribute to future exploitation of an undervalued resource from the Chilean Atacama Desert.

Experimental Section

Plant material collection.

During Flowering Desert of 2017, flowers, leaves and tuberous roots of A. radiata were collected from the Atacama Desert, near to Vallenar City, Chile (28°19’04” S; 70°42’02” W). All plant samples were labeled and stored inside plastic hermetic bags

with wet paper towels to protect them from dehydration. After that, they were placed on the cooler box to be transported from field to laboratory facility. All samples were kept at 4°C to 7°C until processing. Three groups of flower samples were generated, these are yellow, orange and red. Inside those groups, flowers were subdivided by tones, from the lightest to darkest ones. The sample of tuberous roots was divided into cork and pulp respectively, and all together with plant leaves were stored until further analyzed. All plant material was collected by Mr. Pablo Morales, and identified by Mr. Mauricio Cisternas, Biologist and Botanist, Curator of the Herbarium of the National Botanical Garden (JBN), Viña del Mar City, Chile. Samples of this material were deposited in that herbarium under voucher JBN 3450.

Extract preparation

Lyophilized plant material (1g) was mixed with 3 mL of methanol and sonicated for 20 min. The homogenate was centrifuged at 8000 rpm for 5 min. The supernatant was collected and the residue was extracted at the same conditions twice. Supernatants were combined, evaporated to 1 mL under a nitrogen stream. The final extract was filtered through 0.45 µm syringe filters and storage at -20 °C until analysis.

Total Polyphenolic Content (TPC)

The total phenolic content was evaluated using the Folin-Ciocalteu method [51]. 200 µL aliquots of the sample were mixed with 50 µL of Folin-Ciocalteu reagent, 150 µL of sodium carbonate (Na_2CO_3, 20%) and 600 µL of ultrapure water. The samples were let stand at dark for 30 min at room temperature, and the absorbance was measured at 765 nm (Agilent 8453 Spectrophotometer; UV-Visible ChemStation Software Agilent Technologies Rev.A.10.01 95-03). The results were expressed as gallic acid equivalents (mg GAE/g FW).

Total Flavonoid content (TFC)

Total flavonoids of extracts were determined based on $AlCl_3$ reaction [52]. The extract (1 mL) was mixed with aluminum chloride 2% in ethanol, and its absorbance was determined at 420 nm after 1 h. The results were expressed as equivalents of quercetin (mg QE/g FW)

Total Anthocyanin Content (TAC)

The pH differential method [53] was employed for quantifying the TAC in samples, using cyanidin-3-glucoside (CG) as standard. Briefly, 200 µL of properly diluted ethanol extract samples were mixed with 1.8 mL of either 25 mM HCl/KCl (pH 1.0) or 0.4 M acetic acid/sodium acetate (pH 4.5) solutions. The absorbance of the solutions was measured at 520 nm and 700 nm. The results were expressed as equivalents of quercetin (mg eq CG/g FW)

ABTS (2,2-azino-bis 3 ethyl benzothiazoline-6-sulfonic acid) radical cations scavenging activity

1450 mL of ABTS reagent solution was mixed with 50 µL of plant extract [54]. The solution absorbance was measured at 732 nm after 30 min in the dark. ABTS reagent was prepared as follow: 19,4 mg of $K_2S_2O_5$ was dissolved in 20 mL of 7mM ABTS cation; 1 mL of the resulting solution was diluted with methanol until the absorbance of 1,1± 0,02 at 732 nm. A calibration curve was calculated with Trolox.

Ferric reducing antioxidant power (FRAP)

The antioxidant activity was determined by the capacity of iron reduction [55]. 200 µL of plant extracts were mixed with 1.8 mL of FRAP reagent, after 15 minutes in the dark the absorbance was measured at 593 nm. FRAP reagent was prepared as follow: 25 mL of acetate buffer, 2.5 mL TPTZ solution (10 mmol/L of TPTZ in HCl 40 mmol/L) and 2.5 mL of 20 mM $FeCl_3*6H_2O$. A calibration curve was calculated with known solutions of $FeSO_4*7H_2O$.

HPLC-MS/MS analysis

Quantification was performed in a Triple Quad™ 4500 System coupled with an Eksigent Ekspert Ultra LC 100-XL system. Chromatographic separation was achieved in LiChrospher 100 RP-18 end-capped column (125 mm x 4mm id, 5µm) at 40 °C with a mobile phase of 0,1% formic acid (A) and methanol (B). Mobile phase was programmed as follows: 0 – 1 min, 15% B;

1 – 17 min, 15-100 % B; 17-21 min 100-100% B; 21-22 min, 100-15 % B; 22-25 min, 15-15% B; at a 0,5 mL/min flow rate, with injection volume of 10 µL. Electrospray ionization was performed in positive mode. Fragmentor voltage and collision energies were optimized for each analyte during infusion of the pure standard, and the most abundant fragment ion was chosen for the selected reaction monitoring. Quantitative analysis was carried out using multiple reaction monitoring (MRM) mode, using the first transition for quantification and a second transition for identification purpose. For the proposed method, the most intense characteristic MRM transitions were chosen for each analysis. For polyphenolic compounds, flavonoids and abscisic acid commercial standard (Sigma-Aldrich) were used for their identification and quantification.

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Author Contribution Statement

G.C. designed experimental work. P.M. conducted fieldwork, collection, and identification of the samples. A.G. conducted plant extraction and chemical analysis. All authors contributed to data analysis, writing the manuscript and approved the final draft.

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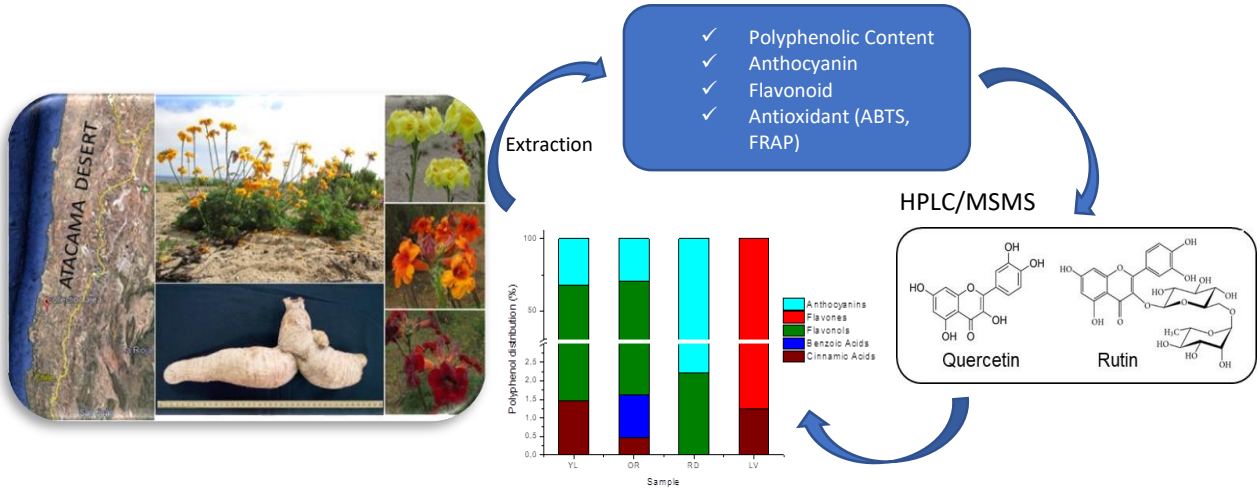
Graphical Illustration



Twitter Text

First study about polyphenolic compounds composition and distribution in *Argylia radiata* extremophile plant. Rutin, quercetin and caffeic acid were the most abundant compounds in organs, they are related to plant stress responses.