DIPLOMA THESIS

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BUDAPEST

Acclimatization of *in vitro* propagated *Vriesea splendens* ‘Fire’ on different substrates

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ABBREVIATIONS

B	bark
C	coco coir
M	moss
P	perlite
T	turf
V	vermiculite
BC	bark + coco coir
BM	bark + moss
BP	bark+ perlite
BT	bark + turf
BV	bark + vermiculite
CM	coco coir + moss
CP	coco coir + perlite
CT	coco coir + turf
CV	coco coir + vermiculite
MP	moss + perlite
MT	moss + turf
MV	moss + vermiculite
PT	perlite + turf
PV	perlite + vermiculite
TV	turf + vermiculite

1. INTRODUCTION AND OBJECTIVE

Vriesea splendens 'Fire' is a variety of flowering plant that belongs to *Bromeliaceae* family from the tropical forest in South America. Thanks to its striped leaves and bright inflorescence with red bract and yellow flowers, it is an ornamental plant very appreciated in Europe where it was introduced from the 19th century. Due to the market demand, the conventional methods of propagation are not enough and micropropagation seems to be an alternative to increase the number of individuals in a homogenous stage, and avoid pathogen attacks. After this *in vitro* propagation under laboratory conditions, the next and last step is the acclimatization, where the plants are exposed to a new environmental condition including the different type of substrate such as turf, perlite, coco coir, sand and other combinations of them.

My aim in this study is to evaluate the acclimatization of *Vriesea splendens* 'Fire' plants on different substrates through the measurement of plant features as plant height, fresh plant weight, leaf length and width, root number and length, total chlorophyll, carotenoid content, and survival rate to acclimatization.

2. LITERATURE REVIEW

2.1. Taxonomic characteristics

Vriesea splendens is native from South America, exactly from tropical forests located at Trinidad, French Guiana, Guyana, Suriname and Venezuela. This species was first time published in 1850 (Van Houtte, 1850) and has one synonym as *Tillandsia splendens* Brongn. This species belong to the *Angiosperms*, to the *Bromeliaceae* family, a high genus diverse family with 58 genera and around 3000 species (Stevens, 2016). From all these species *Tillandsioideae* subfamily, has 9 genera with 1220 species including *Vriesea splendens* (Luther, 2008, Gomes-da-Silva & Souza-Chies, 2018). To sum up, the next taxonomic description (Tropicos, 2021):

- Kingdom: *Plantae*
- Clade: *Angiosperms*
- Class: *Equisetopsida* C. Agardh
- Subclass: *Magnoliidae* Novák ex Takht.
- Superorder: *Lilianae* Takht.
- Order: *Poales* Small.
- Family: *Bromeliaceae* Juss.
- Subfamily: *Tillandsioideae*
- Genus: *Vriesea Lindl.*
- Species: *Vriesea splendens* (Brongn.) Lem.

2.2. Morphological characteristics

The genus *Vriesea*, *Tillandsia* and *Guzmania* are the most morphologically diverse even though they are genetically homogenous, this is because they have developed different strategies to occupy different niches including the top of the trees (epiphyte life form), for example (Vervaeke et al., 2004).

One characteristic of the most *Bromeliaceae* and also for *Vriesea* is the growth in rosette of leaves, forming a natural water tank. This structure is important because it allows the plants to have access to water and nutrients for a prolonged moment, and sometimes gives access to the organic matter decomposition process (Benzing, 1976). It is complemented with the presence of trichomes or absorbing scales, proper in all the members of the *Bromeliaceae* family, at the base of the leaves, which absorb the nutrients and water from the natural water tank reservoir, thanks to the difference of water potential between the leaf's cells and the nutrient reservoir (Benzing, 2005). The leaves show xeromorphic structures (thick cell walls, parenchyma for water-storage, air-lacunae and absorbent trichomes) that evidence an ancestral dry habitat and with a recent establishment at rainforests, wetter habitats (Faria et al., 2021).

Despite the common functionality of leaves in *Bromeliaceae* family, the shape and color of *Vriesea splendens* is particular, with colorful smooth leaves with green and dark green horizontal stripes forming a roseate (Vervaeke et al., 2004). They usually have a high ratio of the leaf surface and fresh weight (Vanhoutte et al., 2017). At the base of the leaves, it produces shoots to future individuals. They have wick roots, which serve as support to the substrate, an essential characteristic because they are epiphytic. The inflorescence has long lived red bracts and it is tubular and with a chromatic variation of yellow, white and greenish. The pollen grains are elliptic, plane-convex, and monosulcate, which means they only have a germ pore (Halbritter 1992). The seeds are thin and wind dispersed with low germination capability (Mercier & Kerbauy, 1995). All these features make *Vriesea splendens* aesthetically different to be a house pot plant especially in Europe, but to cover this demand, a quick and high-quality propagation is needed.

2.3. Propagation of *Vriesea* genus

2.3.1. Sexual propagation

The bromeliads sexual propagation is sensitive to environmental factors like substrate type, temperature, light and humidity (Silva & Varassi, 2016). For example, the bromeliads in the shade produce less seeds per fruit than sun exposed plants (Scrok & Varassin, 2011).

The principal step for the sexual propagation is the pollination. The pollinators of *Vriesea* are variable such as pollinated by hummingbirds, bats, bees and butterflies. Regarding to seed dispersion, it is simplest, only by wind (Silva & Varassi, 2016). One factor that could influence the successful rate of pollination is the size of the inflorescence; if it is bigger, it could produce more seeds per fruit (Scrok & Varassin, 2011).

If the environmental conditions are the same, the sexual and asexual propagation can be equal (Silva & Varassi, 2016). However, when the pollinators are not available, the asexual propagation or cloning plays an important role to ensure a fitness.

2.3.2. Asexual propagation

Vriesea has a reproduction, which is monocarpic and clonal at axillary buds in the sheaths of basal leaf (da Costa, 2014). This clonal formation is a strategy that consists in the formation of compact small clonal fragments attached to the mother plant (Sampaio et al., 2002). In this way, the plant is able to retain their original site and increase their area of dominance. After seed dispersion the mother plant starts to die and the clonal can take its place.

2.3.3. Micropropagation

The micropropagation of *Vriesea splendens* have not been broadly studied, but in general, this technique is a massive propagation to protect threatened bromeliad species. This technique has been explored in other ornamental *Vriesea* such as *V. incurvata* (Sasamori et al., 2018), *V. reitzii* (Alves et al., 2006), *V. poelanii* (Hui, 2005). On the other hand, other bromeliads studied due to their economic importance such as *Ananas* (Be & Debergh, 2006; Mhatre, 2007; Zuraida et al., 2011; Scherer et al., 2013; Usman et al., *2013*), *Aechmea* (Huang et al., 2011), *Tillandsia*, *Cryptanthus*. This technique, in general terms, consists in propagating the seeds, inoculating them in rich media and after 8-10 weeks (depending on *Vriesea* species) transfer them to another medium, removing the leaf segments from the basal region of young shoots to new media (Alves et al., 2006). Finally, the morphogenetic and physiological parameters should be measured by the determination of chlorophyll, examining peroxidase activity, the shot number, the plant height, fresh plant weight, root number and survival rate to acclimatization (Ördögh, 2022).

2.4. Requirements of *Vriesea* genus

The *Vriesea splendens* is a kind of rosette with tank-shape leaves that tend to not require optimal nutrient conditions all the time, because they can conserve it for a time between their leaves (Zotz & Asshoff, 2010).

However, when the *Tillandsioideae* plant in horticultural substrates, the soil conditions are absorbed by the roots and satisfy the absorption function, and not only support like happen with the wild epiphyte specimens.

In general, the epiphytic bromeliads tend to colonize the disturbed areas (Hietz et al, 2012). In order to have a good support on the rough branches over the flats increasing the friction (Winkler et al., 2009). One of the requirements of *Vriesea* is the moisture and low light because in the wild specimens tend to live at very shaded tree branches, and if there is moss the germination rate of *Tillandsioides* increases (Hietz et al, 2012).

The nutrient supply comes from the water tank of bromeliads, soil or rain water over the leaves. These nutrient uptakes are higher in the young stage than in adults. In the case of phosphorus, it can be absorbed by the trichomes in the leaves or there is starvation of the older leaves in order to transfer their phosphorus to the younger leaves, through their previous decomposition on soil or water tank (Winkler & Zotz, 2009). For nitrogenous the plants uptake organic and inorganic N forms by the trichomes, but there is a clear preference for $NH4+$ over $NO3-$, sometimes urea can be a potential source for N (Inselsbacher et al., 2007). In the case of potassium, the absorption is also principally by the trichomes and after this approximately 40% of K is recycled on leave decomposition, the same for N (35%) and P (62%) (Winkler & Zotz, 2010). Under commercial and controlled conditions *Vriesea splendens* take nutrients and water for both roots and absorbing trichomes (Vanhoutte et al., 2017), but in humid, shade and in epiphyte life form, they prefer to use the trichomes.

2.5. Biological risk factors for *Vriesea* genus

In a broad sense, the bromeliads are sensitive to insects belonging to *Hemiptera* order, fungus of the class deuteromycetes such as *Fusarium*, and bacteria like *Pseudomonas* and *Erwinia* (Delascio, 1978; Giongo et al., 2019). Due to this continuous exposition to these bacteria, some of the bromeliads have develop antibacterial activity against bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella dysenteriae*, *Salmonella tythimurium* and *Enterobacter cloacae* (Fabri & da Costa, 2012).

There are not too many known diseases and pests reported specifically only in the *Vriesea splendens*, but yes, in the *Vriesea* genus. The larvae *Napaea eucharilla* (*Lepidoptera*) is an herbivory agent for *Vriesea sanguinolenta*, which consumes 4.4% of leaf area in the total population annually, this could change the physiology and population dynamics of these bromeliads (Schmidt & Zotz, 2000). Another negative agent is *Erwinia carotovora*, which has been studied on *Vriesea poelmanii*. It affects the survival during transplantation on propagation, usually appearing due to the excess of moisture (Hui, 2005). During the micropropagation to avoid this kind of damaging it is recommended to have well-ventilated and aseptic workspaces, also control the humidity and excess of irrigation (Hui, 2005; Pardo et al., 2010).

2.6. Importance of *Vriesea* genus

2.6.1. Ecological importance

The bromeliads due to their architecture can be considered microhabitats to other species such as bacteria, insects and amphibians.

The *Vrieseae* genus and in general bromeliads keep biodiversity of bacteria in their leaves and tank water (Giongo et al., 2013; Giongo et al., 2019). Some bacteria could have beneficial effects on *Vriesea* plant growth such as *Pseudomonas* and *Enterobacter* (Ambrosini et al., 2007; Giongo et al., 2019).

Vriesea also is a habitat for insects in their life cycle, especially in the reproductive stage. In just *Vriesea sanguinolenta* there were 65,774 insects in 153 species of *Hymenoptera*, *Diptera* and *Coleoptera* order (Bermúdez-Monge & Barrios, 2011). In addition, the morphological features of the *Vriesea* species such as the size influence in the insect community structure (Bermúdez-Monge, 2010). The insects also are attracted by the inflorescence, for example by the exudate from *Vriesea bituminosa* flower (Monteiro & Macedo, 2014).

Vriesea is also a habitat for vertebrates, such as amphibians, because the rosette leaves create a humid habitat (very important in seasonally regions) which helps to protect against predators, bring an aquatic environment for reproductive stages and also provide insects as food. For example, *Vriesea neoglutinosa* is associated with the population dynamics of frog *Phyllodytes luteolus* (Papp & Papp, 2000). Also, *Vriesea bituminosa* has an association with *Scinax hayii*, where these amphibians also contribute to the nutrition of the plant through their wastes (Romero et al., 2010).

2.6.2. Economic importance

Most bromeliads are focused on the floriculture industry, with some exceptions like pineapple (*Ananas comosus*), and other bromeliads used for fiber extraction for packing or substrate, such as *Tillandsia*. Among the species with more horticultural use are the genera *Aechmea, Ananas, Billbergia, Cryptanthus, Neoregelia* and *Nidularium* (Mercier & Kerbauy, 1997). The bromeliad market is highly competitive and products together, for example, the commercialization of bulbs, rhizomes, tubers and crowns is estimated annually US$ 1,057,865,584 (COMTRADE, 2009). However, in the countries where these *Bromeliads* are native the flower production is not environmentally sustainable and implies some social problems such as worker exploitation and pollution by the industries (Negrelle et al., 2012). Also, the attractive use of these bromeliads put at risk the wild specimens which will need protection of non-controlled harvest with ornamental purpose.

Vriesea splendens, as other species from *Vriesea* genus (*Vriesea carinata*, *V. fenestralis*, *V. hieroglyphica*, *V. imperialis*, *V. saundersii*, *V. tessellata*), are important ornamental plants around the world as indoor or outdoor plant. Due to their success on the market, some hybridization studies have been carried out in order to find innovative shapes and color for the customers. For example, intergeneric hybridization was successful between *Vriesea* and *Tillandsia* forming *Vrieslandsia,* and *Vriesea* and *Guzmania* forming *Vriesmania* (Deroose et al., 2002). Another study of this hybridization was made with *Vriesea michaelii* and *Vriesea nahoumii*, *Vriesea simplex* and *Alcantarea nahoumii,* it suggests that this self-compatibility helps to avoid the self-fertilization maintaining genetic variability. The genetic biodiversity is in danger especially on vegetatively propagated species in ornamental industries, because low genetic variation implies vulnerability to pest or climate change, for example.

2.6.3. Conservation status

The high economic importance of *Vriesea splendens* have attracted the attention of an illegal distribution and commercialization of wild specimens (Pardo et al., 2010). Also, the natural habitat loss and fragmentation of the tropical rain forests, thanks to the deforestation, overexploitation or urbanization (Brook et sl., 2002; Morris et al., 2010) have decreased their number of individuals of *Vriesea splendens*. Then, in some Latin-American countries as Venezuela (LLamozas et al., 2003), Colombia (Betancur & García, 2006) and Brazil (Versieux, 2011; Versieux, 2018) are considered vulnerable and are treated in the red books, list of endangered species for each country.

In order to conserve *Vriesea splendens* there are some strategies that could be applied. In the first place, to conserve the original habitat with wild and alive specimens there, in situ conservation (Braverman, 2014). The other step to conservation is the store of seeds and plant tissue at germplasm bank (de Vicente et al., 2006), which is useful for a long time. On the other hand, the other alternative to conserve is through *ex situ* conservation, which implies *in vitro* conservation, *ex situ* propagation, and acclimatization of *Vriesea splendens* (Engelmann & Engels, 2002; Alves et al., 2006; da Silva et al., 2009; Dal Vesco et al., 2014). Due to the fact, it is an ornamental plant it can be conserved as exotic plant, especially in Europe. However, in these different environmental conditions in which the acclimatization process is required in order to achieve an optimal plant development.

2.6. Acclimatization studies on *Vriesea* genus

The studies of acclimatization on the *Vriesea* genus are the last step of research techniques of micropropagation. These studies are principally focused on increasing the production of individuals of certain species of *Vriesea* with great ornamental importance, because they are in danger of illegal harvesting in wild states (Table 1).

One important and relatively deep study species is *Vriesea reitzii* from brazilian atlantic forest biome because it has high ornamental value. It is important to improve *in vitro* micropropagation techniques and evaluate their results in different acclimatization processes. Also, one particular aspect of micropropagation is the interest in the nodular induction culture (Rech-Filho et al., 2005; Rech-Filho et al., 2009; Dal Vesco et al., 2014; Alves et al., 2016). The treatments tested with this species have been with hormones such as α-naphthaleneacetic acid (NAA), 6-benzilaminopurine (BAP) or thidiazuron (TDZ), gibberellic acid (GA3), showing the gibberellic acid a better survival rate in the acclimatization phase than the other *in vitro* treatments (Dal Vesco et al., 2014; de Resende et al, 2016).

The literature shows lack of comparative studies on acclimatization conditions, especially on substrate treatments on *Vriesea*. One variable in the substrate as the pH can evidence and be crucial for an appropriate development of the plants. For example, in *Vriesea philippocoburgii* changing the substrate pH at different concentrations of sulphur, even though the survival rate was 100% for all, there was a dramatic damage on some plants depending on the treatment (Kämpf et al., 2009). However, the 0.5 g sulphur/L substrate provided the best result for the plants at this stress.

Some studies on the micropropagation suggest certain conditions that can improve the acclimatization success. One factor is the moderate percentage of sucrose during *in vitro* culture that can promote root growth, number of leaves, highest fresh mass and lowest contents of chlorophylls and carotenoids, strengthening plants for acclimatization (Freitas et al., 2015; Sasamori et al., 2018; Sasamori et al., 2019). One recent study shows the positive effect of hormones as kinetin (KIN), indole-butyric acid (IBA) and naphthaleneacetic acid (NAA) that contribute to the root formation *in vitro* cultivation on the survival rate in acclimatization (Ördögh, 2022). Another factor that can influence the acclimatization is the temperature at *in vitro* conditions, for example the leave length at 15 °C were smaller than those plants at 28 °C, giving the leave length an advantage of more area to transpiration and photosynthetic activity (Pedroso et al., 2010).

3. MATERIALS AND METHODS

3.1. Plant material

The seeds of *Vriesea splendens* 'Fire', a smaller sized cultivar with 20-25 cm leaves and 30-35 cm flowers stalks (Tillyné & Honfi, 2008) were harvested from a motherplant and were used for *in vitro* studies in the laboratory of the Department of Horticultural and Dendrology, Hungarian University of Agriculture and Life Sciences. After multiplication and rooting, *in vitro* specimens were pre-treated, and then acclimatized in one of the greenhouses and laboratory of the department.

3.2. Pre-treatment

The pre-treatment consisted on separating the different *in vitro* shoots product into Erlenmeyer flasks of 100ml with Murashige and Skoog (1962) basic media with 6.5 g/l agar, 20 g/l sucrose (Figure 1).

Figure 1. Agar medium in flasks with *Vriesea splendens* 'Fire' (thereinafter: *V. splendens*) shoots

This process was done in sterile area of laminar flow cabinet to avoid contamination (Figure 2). In each flask were equally distributed 3 shoots of *V. splendens* of 0.5-2 cm (Figure 3). In total, there were more than 700 individuals that were growing there during 3 months at 20-25 ^{o}C under 16-hour lighting/day, 10 Watt/m^2 energy with 1500-2000 lux light intensity, lamp type: fluorescent lamp (T8 Polylux XL, 30 Watt, cool and warm white), Figure 4.

Figure 2. Sterilized materials, tools (left), and laminar flow cabinet as workspace (right) to avoid contamination

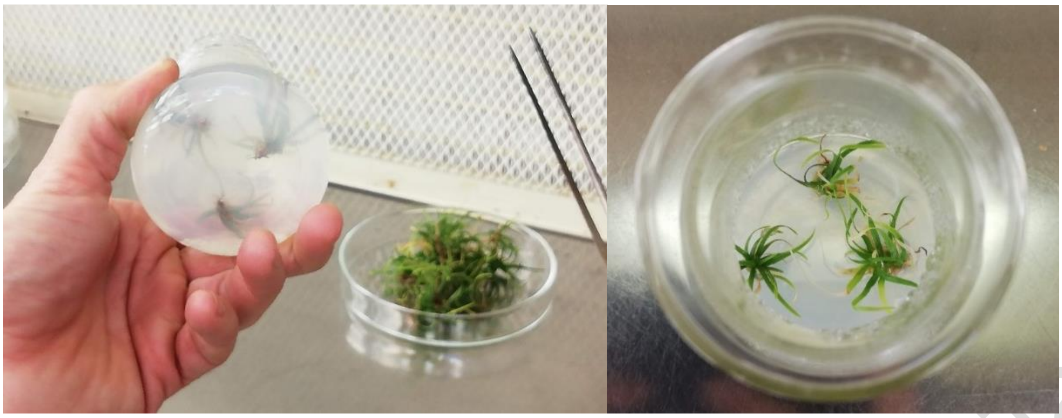

Figure 3. *V. splendens* shoot distribution in a medium in flask, bottom (left) and apical (right) perspective

Figure 4. Pre-treatment: *in vitro* stock of *V. splendens* in the culture room of the micropropagation laboratory of the Department of Floriculture and Dendrology

3.3. Selection of study groups

After the pre-treatment, plants were washed in bowl with tap water to put out the growing media from the roots (Figure 5, left). These plants usually developed more shoots, which were separated using tweezers trying to conserve just one main shoot (Figure 5, right). In some cases, the plant had an important shoot attached, which was conserved because the separation of shoots and of their roots can put in risk the survival of the plant (Figure 6).

Figure 5: After pre-treatment: cleaning *V. splendens* plants (left), and separated main shoots (right)

Figure 6: Close view of a *V. splendens* plant separation (left side) from secondary shoots (right side)

3.4. Acclimatization in one step, in greenhouse (first acclimatization)

The cleaned, separated plants were transferred to the greenhouse, and planted into plug tray (consists 104 cells) filled with different substrates. The principal substrates are the most commonly used turf (baltic or "white" turf), vermiculate, perlite, coco coir, pine bark and moss (marked as terrarium live forest moss). First, turf substrate is partially decayed organic matter from plant material accumulated by soil conditions such as waterlogging, low oxygen, low nutrient content and high acidity. Then, provide nutrients and protection to the plant from high organic matter (Atzori et al., 2021). It is a good substrate because can support the growth of model plants as *Arabidopsis thaliana* with presence or absence of microorganisms (Kremer et al., 2021). Second, the vermiculite substrate is a mineral combination of iron and magnesium silicates that provide structure and water retention. Also vermiculate facilitate the natural growth of mycorrhizas, which helps to nutrient supply to plants and increase survival (Sato et al., 2020). Third, perlite is a product of volcanic rock exposed to high temperatures and pressures forming a foamy amorphous white fragment. This substrate improves porosity and aeration capacity, increasing the content of water capacity (Markoska et al., 2018). Fourth, coco coir substrate comes from the fibers inside of coconut husk that include minerals such as potassium zinc, iron, manganese and copper. Due to the lignin content, it helps to the construction of lignocellulose in plants (Sangian & Widjaja, 2017). Fifth, pine bark, is the woody cortex from the *Pinus* species. This is highly acidic and not increase the water content, but yes, the moisture (Altland et al., 2018). Sixth, moss due to their hyaline cells in the leaves can accumulate water and provide humidity to the plant if it is used as substrate (McCarter & Price, 2014).

The plants were separated in groups of 30 individuals in all substrates, in total 630 plants were tested (Figure 7). The treatments used variate on the percentage of content of the substrate and the pair possible combinations of them. At the end, there were 21 different kind substrates in the plug trays (Figure 8):

1. 100% turf
2. 100% vermiculite
3. 100% perlite
4. 100% coco coir
5. 100% pine bark
6. 100% moss
7. 50+50% turf + vermiculite
8. 50+50% turf + perlite
9. 50+50% turf + coco coir
10. 50+50% turf + pine bark
11. 50+50% turf + moss

12. 50+50% vermiculite + perlite
13. 50+50% vermiculite + coco coir
14. 50+50% vermiculite + pine bark
15. 50+50% vermiculite + moss
16. 50+50% coco coir + perlite
17. 50+50% coco coir + pine bark
18. 50+50% coco coir + moss
19. 50+50% perlite + pine bark
20. 50+50% perlite + moss
21. 50+50% moss + pine bark

Figure 7. *V. splendens* plant distribution indifferent study groups for different substrates (left) and assignation of each study group of plants to one substrate type (right)

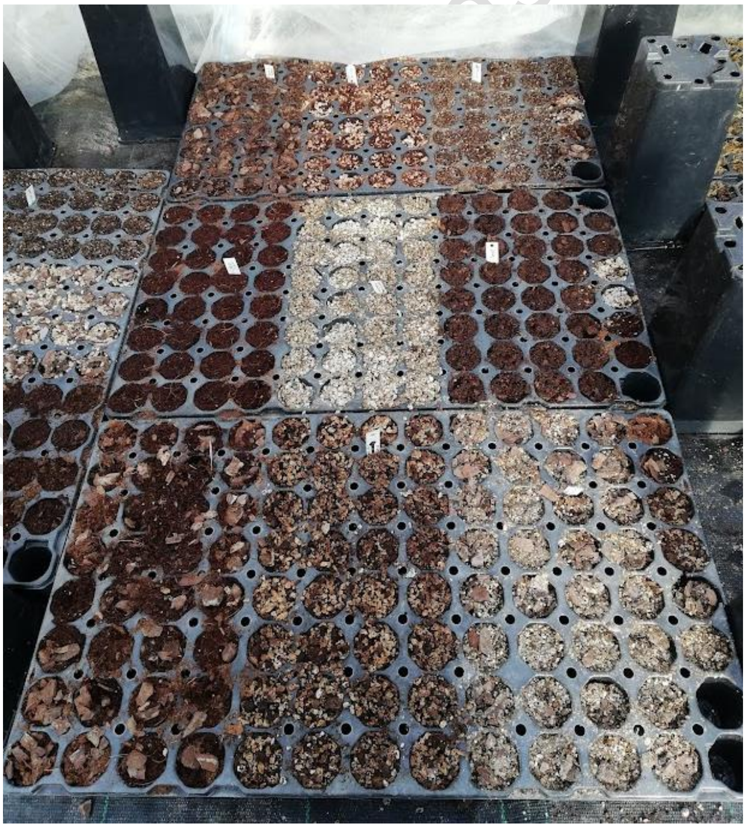

Figure 8: Different substrates types already in the trays, before planting the *V. splendens* groups

After planting, during the first 3 weeks a fiber cover was necessary to help to maintain the humidity (Figure 9). Thereafter all plants were grown under greenhouse conditions with equal irrigation for all substrate treatments (Figure 10), under natural light at a temperature range of almost the same as in the laboratory (18-27 ^{o}C), maintaining the temperature conditions of the pretreatment. After 5 months, the physical and physiological features were measure, thus, the total duration of the acclimatization has the same time.

Figure 9. Fiber cover for the first 3 weeks to keep the sensitive plants in optimal humidity level

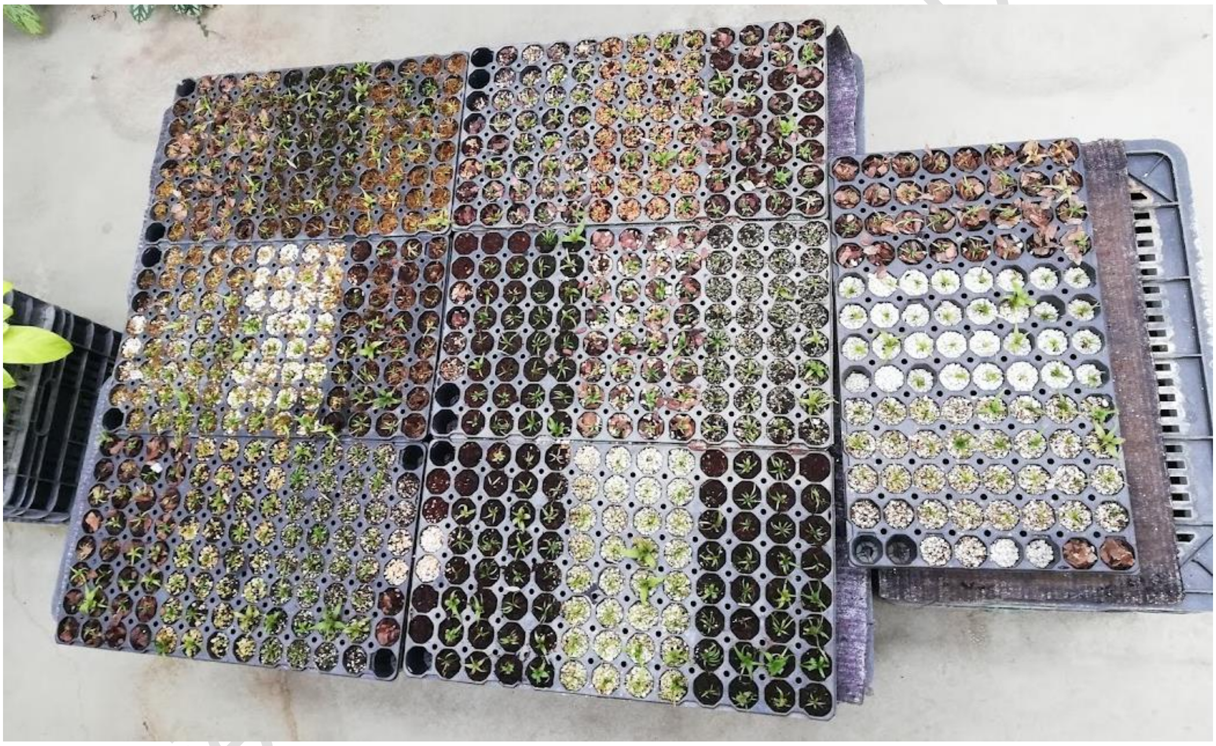

Figure 10. *V. splendens* plants in the 21 different substrate treatments (first acclimatization)

3.5. Acclimatization in two steps, in laboratory and in greenhouse (second acclimatization)

For this trial, coconut, turf, perlite and vermiculite substrate and their respective combinations were used (Figure 11 and Figure 12). As the first step, cleaned, separated shoots were planted in 200 ml sized glass jars filled with 10 kinds of substrates (see below) and covered by two layer of foil pack. During the next 3 months, all these stocks were kept on the shelves of the department laboratory culture room, with the same lighting, temperature conditions detailed as pre-treatment in chapter 3.2. As the second step, plants (which has already developed a small root ball) transferred to the greenhouse, carefully placed them into 104 cell plug trays filled with the same substrates that was used previously, and 2 months later, survived specimens were examined based on the same morphological and physiological parameters just like in the case of the first acclimatization. The duration time was also 5 months, including both steps.

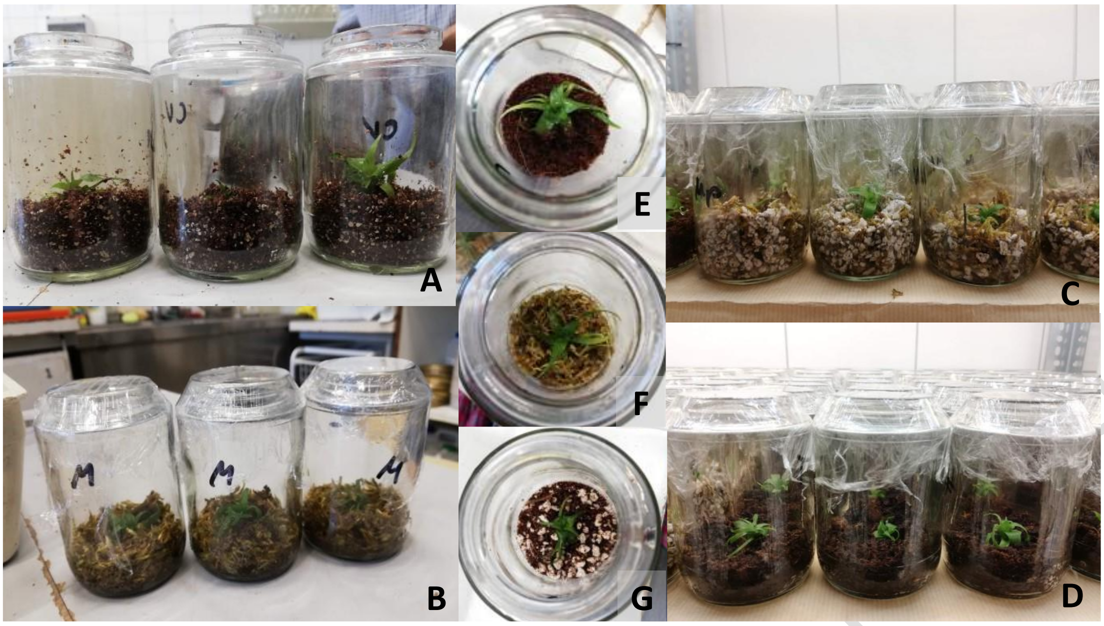

Figure 11. *V. splendens* individuals in glass jars, grown under laboratory conditions. A: coco coir + vermiculite, B: moss, C: perlite + moss, E: turf (top-view), F: moss (top-view), G: perlite + coco coir (top-view)

Figure 12. *V. splendens* individuals in glass jars, grown under laboratory conditions. A: glass jars on shelves with controlled light. B: all vessels were covered by fold pack (close view). C: planted glass jars with moss + perlite (top-view), D: empty glass jars with perlite (top-view)

Due to preliminary results of the first acclimatization, pine bark and moss were inappropriate for the plants. The substrate mixes with pine bark and especially pure (100%) bark presented low survival rate only around 50 %

of the plants did not growth. Moss has similar negative effects. These kinds of substrates are usually applied with others like sand or turf to provide humidity and increase the bulk capacity (Altland et al., 2018).

30 plants will be placed in each of the 10-substrate, in total 300 plants were used:

1. 100% coco coir
2. 100% turf
3. 100% perlite
4. 100% vermiculite
5. 50% coco coir+ 50% turf
6. 50% coco coir + 50% perlite
7. 50% coco coir + 50% vermiculite
8. 50% turf + 50% perlite
9. 50% turf + 50% vermiculite
10. 50% perlite + 50% vermiculite

3.6. Plant features measurement

The morphological and physiological features were measured after the pre-treatment and before the end of acclimatization either in the first and the second one.

3.6.1. Morphological features

The first physical parameter examined was the number of shoots manually counted. Secondly, the plant height was measured in mm with a standard rule, and the longest-widest leaf in vertical direction per shoot (Figure 13, left). In addition, for the fresh plant weight, an analytical balance was used in grams (Figure 13, right).

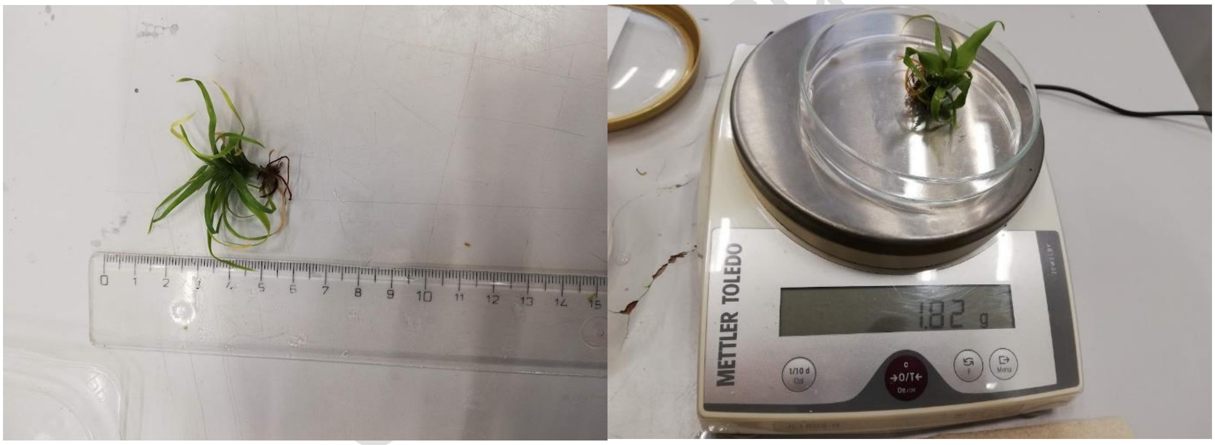

Figure 13: Leaf, root length (left), fresh weight (right) measurement of a *Vriesea splendens* plant

In the below ground plant, the root was evaluated in the number of roots and length. The length is calculated by the longer and main root until the basal part of the leaves.

3.6.2. Physiological features

Total chlorophyll (chlorophyll-a + -b) and carotenoid content were determined, and around 100 mg of leaf sample were measured from each shoot in different groups (according to the substrates). The pigments were extracted by mixing crushed leaves at a ceramic mortar with 0.5 g of quartz sand and 10 ml acetone at 80% (Figure 14). Then, suspensions were refrigerated for 24-hour at + 4 ^{o}C period. Finally, the absorbance was measured at 644, 663 and 480 nm wavelengths by GeneSys VIS-10 (Thermo Fisher Scientific Inc., USA) spectrophotometer (Figure 15).

To calculate the leaf pigment concentration (µg g-1) for chlorophyll, it is needed the volume of tissue extract (V), 10 ml; fresh weight of tissue (w), 0.1 g; and absorbance (A) at 644 nm and A663 nm. These values were used in the next formula: $(20.2 \times A_{644} + 8.02 \times A_{663}) \times V/w$ (Arnon, 1949).

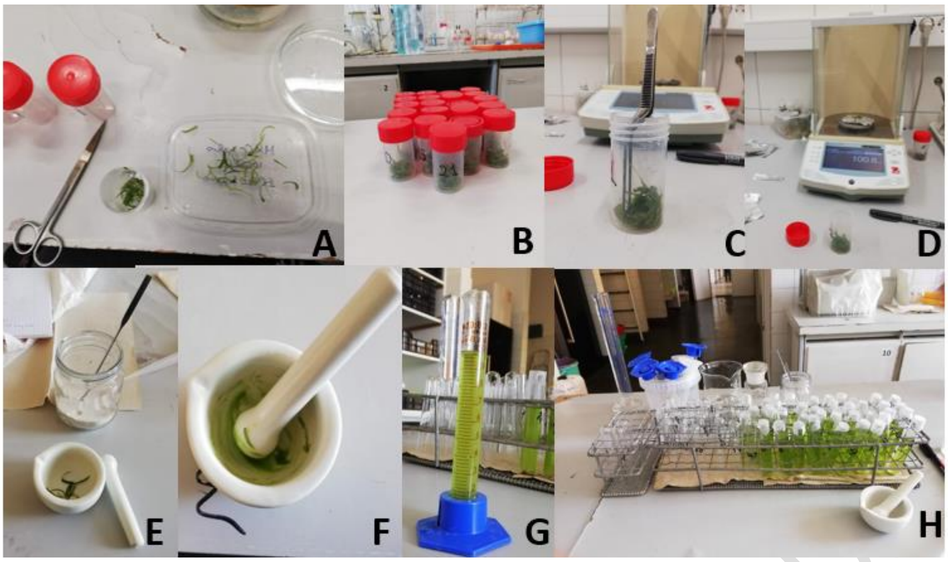

Figure 14. Pigment extraction from *V. splendens.* A: collection of leaves from each plant group. B: storing of leaf samples separated in groups. C: measure of leaf sample. D: analytical balance used for weight measure of leaves. E: leaves trituration with sand and F: with acetone. G: 100 ml extract. F: tubes with the extracts, covered by Parafilm® stretch foil to avoid evaporation

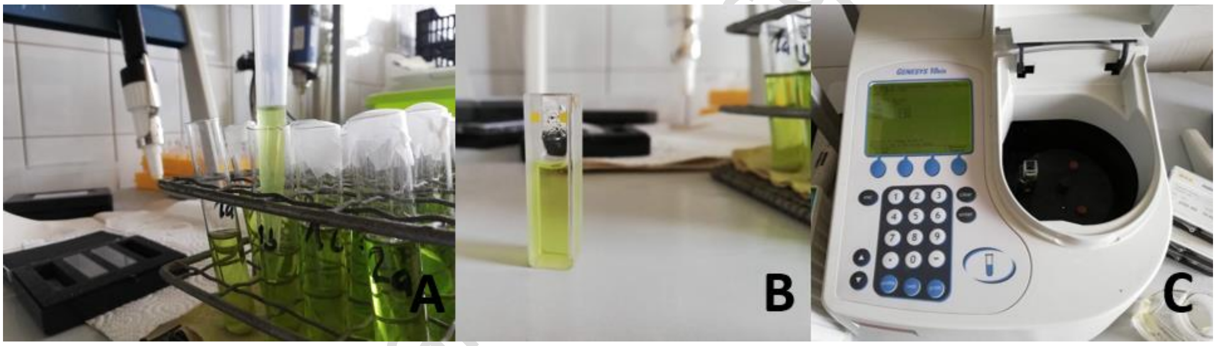

Figure 15. Leaf pigment measure by spectrophotometry. A: cube charge by pipette. B: cube charged for measure. C: measure of 644, 663 and 480 nm wavelengths by GeneSys VIS-10 spectrophotometer

Determining the carotenoid concentration in the leaves, it is also needed the volume of tissue extract (V), 10 ml; fresh weight of tissue (w), 0.1 g; and absorbance (A_{480}) at 480 nm. These values are included in the mathematical formula $(5.01 \times A_{480})/w$ (Arnon, 1949).

The survival rate of acclimatization was calculated as the percentage of plants that are still alive after 5 months of both acclimatization. This measure will be done after the finalization of the experiments.

3.7. Statistical analysis

Data per each shoot in each treatment is organized and cleaned in excel file: number of shoots, plant height, fresh plant weight, leaf length, leaf width, root number, root length, total chlorophyll and carotenoid content, survival rate of acclimatization. These data were processed on SPSS Statistics 23.00 software (IBM Corp., USA).

To calculate the statistical differences of variance between treatments I used one-way analysis of variances (ANOVA). Means were compared using Tukey HSD (Dunn, 1961) depending on whether the homogeneity of variances was accepted or violated, at a 5% probability level ($p < 0.05$).

4. RESULTS

4.1. Morphological and physiological results of the first acclimatization

4.1.1. Morphological results of the first acclimatization

In the case of the shoot number (Figure 16), it looks rather uniform for all substrates and the difference from the initial and final stage was not too large. The highest shoot number was obtained on moss + perlite substrate (1.57 pcs) and this substrate effected the biggest difference between the initial and final stage. Additionally, moss + perlite resulted significantly higher final shoot number than the lowest values (mostly: one shoot) belongs to bark, moss and bark + moss. From these, bark + moss and perlite + bark were the substrates, which generated less units of shoots from the beginning of the experiment.

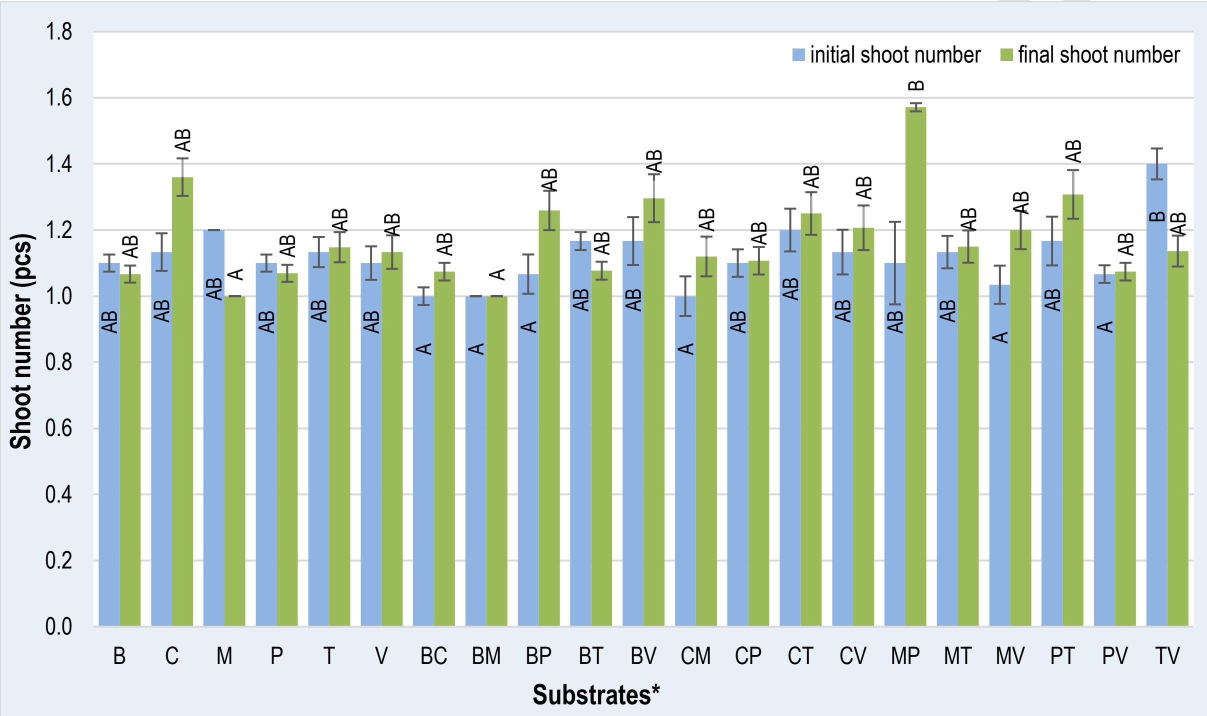

Figure 16. Shoot number means per substrates in the first acclimatization (*the name of the substrates were represented in the Abbreviations on page 4 – in the case of all graphs)

Regarding the second morphological variable, plant height (Figure 17), in contrast to the shoot number, there was a strong difference among the height at the beginning and at the ending of the experiment. The highest plants growth (not showing a significant difference) was on coco coir substrate (60.80 mm), followed by coco coir + turf (58.5 mm) and coco coir + vermiculite (55.5 mm). On the other hand, the lowest plant height was on bark (30.13 mm) and moss substrates (33.06 mm). The unique significant difference was presented on bark + perlite mixed substrate with 51.4 mm. Height differences of acclimatized plants grown in bark, moss, coco coir + turf and coco coir were shown on Figure 18.

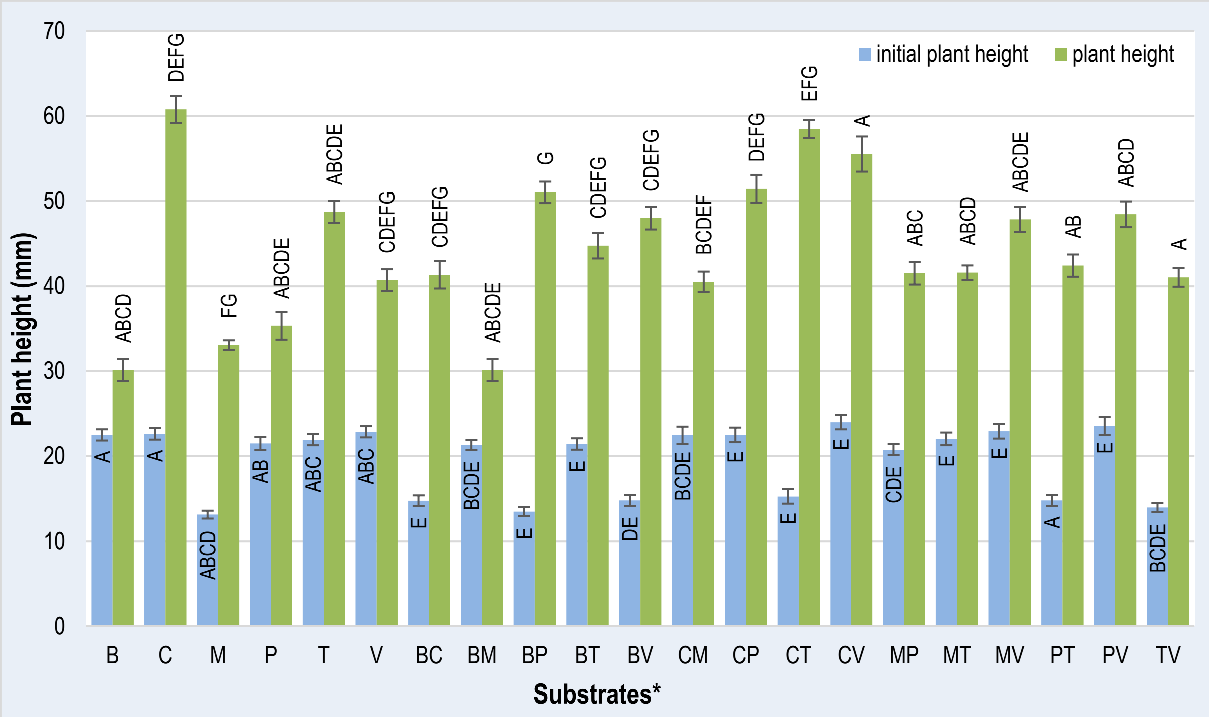

Figure 17. Plant height means per substrates in the first acclimatization

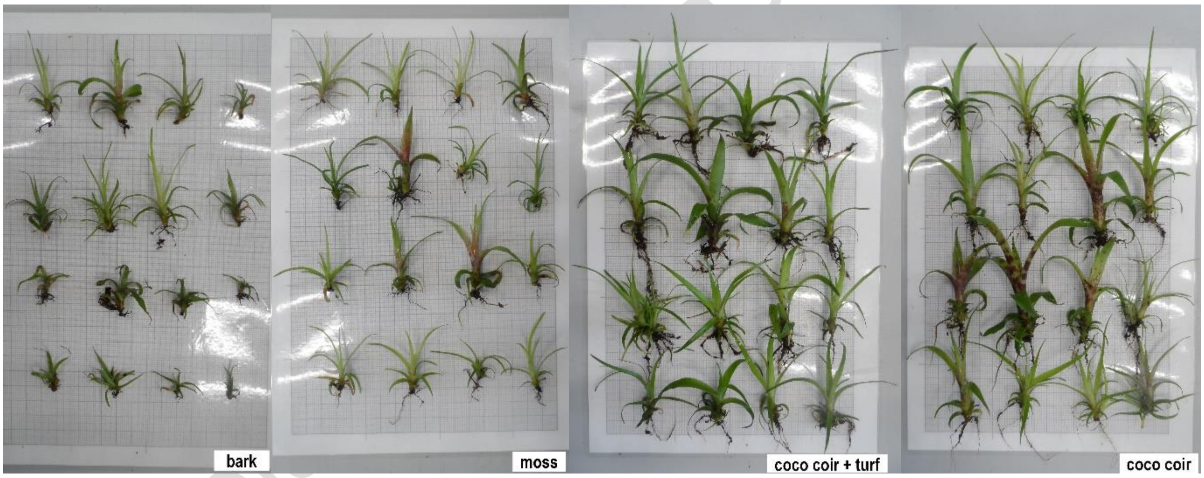

Figure 18: Plants grown in bark, moss has the lowest height, and coco coir, coco coir + turf resulted the highest specimens

According to the third character, fresh plant weight (thereinafter: plant weight), the initial weight presented the same average at the same time that after the experiment the plant weight notably increased (Figure 19). Some exceptions, not significant different were the bark (0.36 g) and perlite (0.39 g) substrates which differed a bit from the initial stage, showing the lowest final weight. The biggest three plant weights belonged to coco coir + vermiculite (1.18 g), coco coir + turf (0.99 g) and coco coir + perlite (0.08 g), from whom the result of coco coir + vermiculite was significantly higher than the others. In the case of the latter substrate, one of the heaviest plants (with typical purple leaf patterns) was shown on Figure 20.

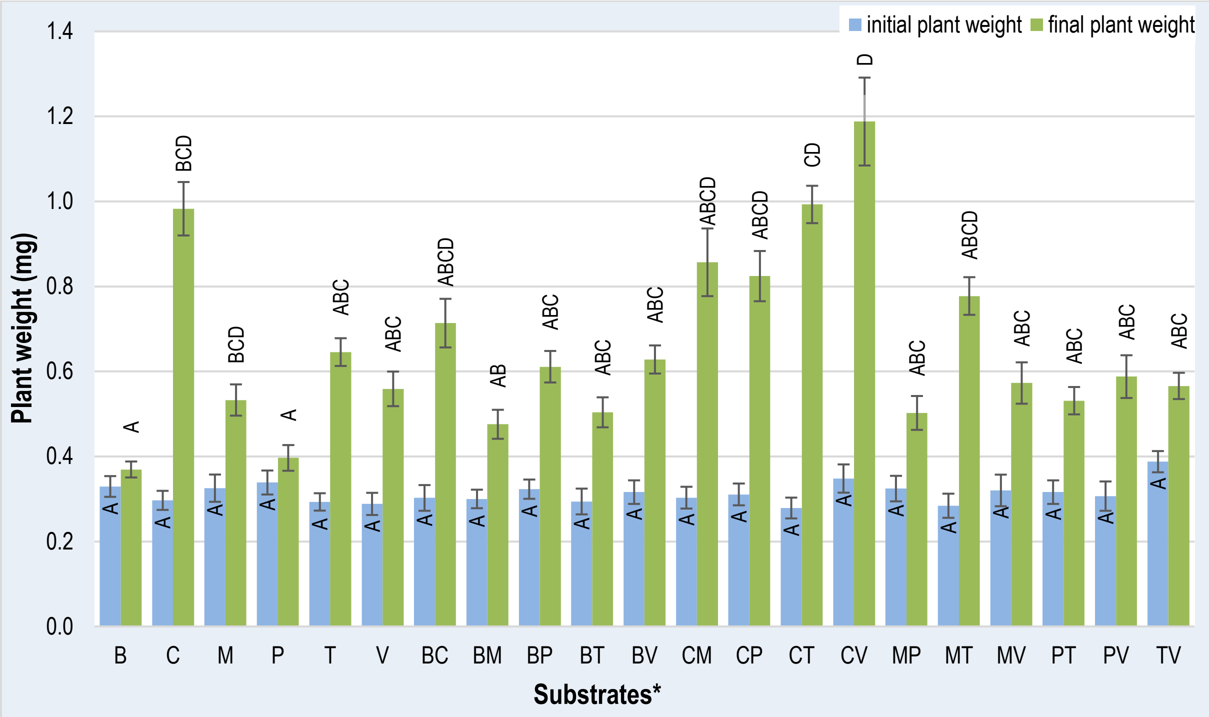

Figure 19. Plant weight means per substrates in the first acclimatization

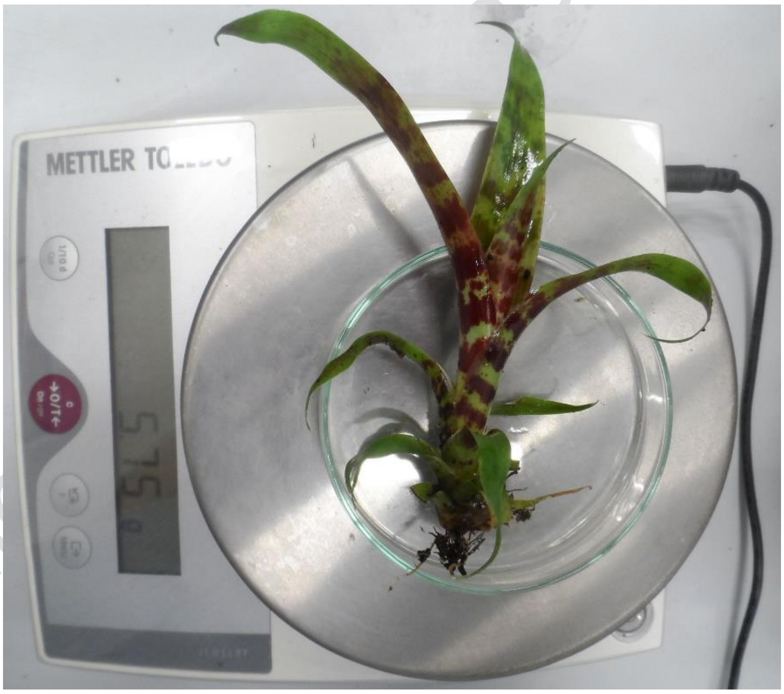

Figure 20: Coco coir + vermiculite resulted the heaviest plants (one of them reach almost 5.8 g)

The fourth morphological variable was the leaf length (Figure 21). The initial and the final length values changed in each substrate. The longest leaves developed on coco coir + turf (74.46 mm), coco coir (71.86 mm) and coco coir + vermiculate (72.40 mm), from which unique significant different was for coco coir + turf substrate. Belonging for the lowest leaf length variable, plants evolved smaller leaves were significant different on bark substrate (37.67 mm).

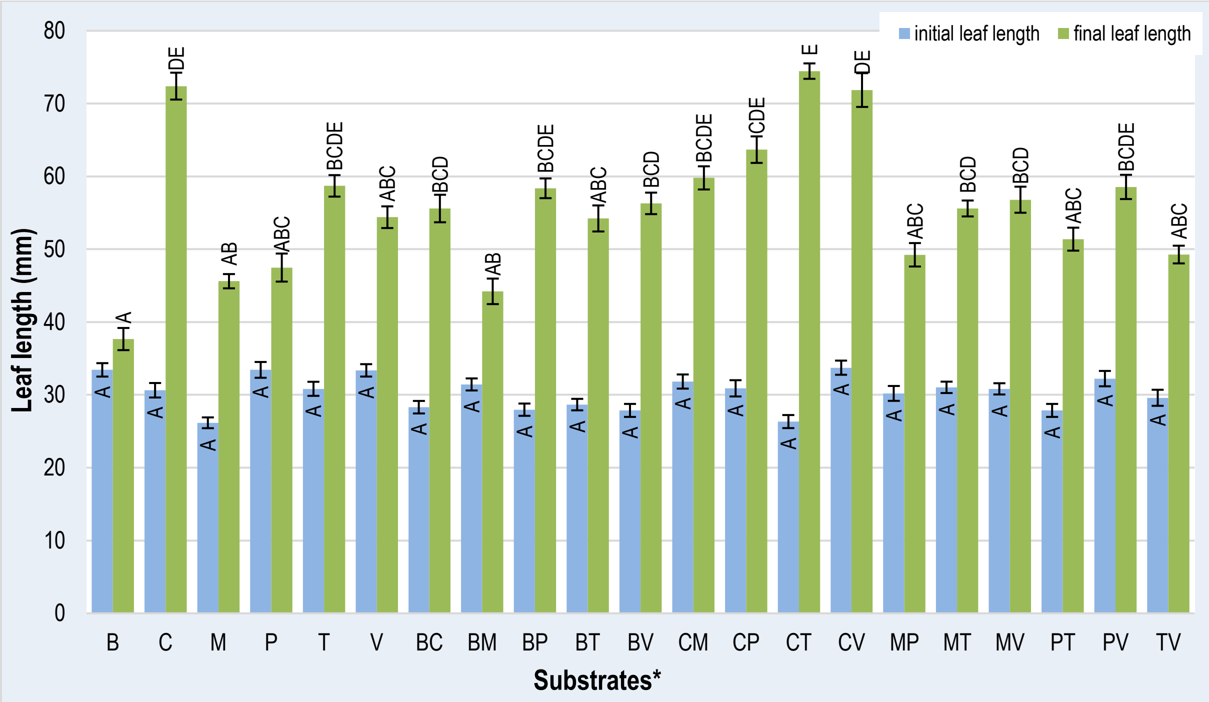

Figure 21. Leaf length means per substrates in the first acclimatization

With regard to the fifth feature, the leaf width values (Figure 22) shown the same initial averages, but distinct final widths in each substrate type. The widest leaves were developed in the case of coco coir (6.88 mm) together with other results from coco coir+ turf mixture (6.18 mm) and moss (5.89 mm). All these substrates resulted significantly higher values than the smaller leaves in width belonged to the bark (2.53 mm), bark + moss (2.74 mm), vermiculite (3.17 mm) and moss + turf (3.2 mm). Moreover, compared to the initial leaf width sizes, particularly smaller final values were detected on bark, bark + moss.

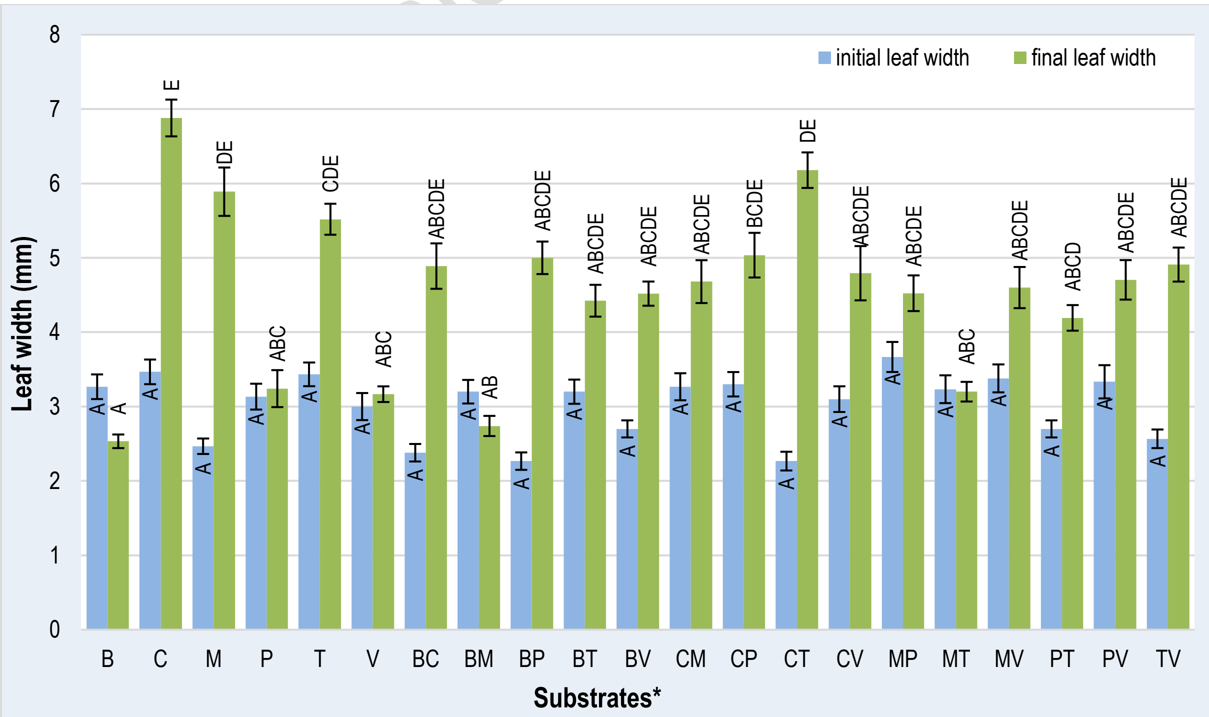

Figure 22. Leaf width means per substrates in the first acclimatization

The sixth examined physical parameter was the root number, and in contrast to the other features presented in the previous graphs, the initial means were higher than in the final stages, in every group (Figure 23). The less change in root number happened on bark + moss which was almost the same than the beginning and the strongest change was registered on bark + vermiculite (4.62 pcs), decreasing more than the half of initial root number. At the end of the experiment, the biggest number of roots was observed on moss (5.8 pcs). Turf (5.44 pcs), coco coir + turf (5.43 pcs), moss + vermiculite (5.48 pcs), perlite + vermiculate (5.44 pcs) also efficiently stimulated root development, which were not significant different among them. Additionally (and compared to the moss), significantly the fewest roots were resulted by coco coir + moss (3.24 pcs) and moss+ turf (3.05 pcs).

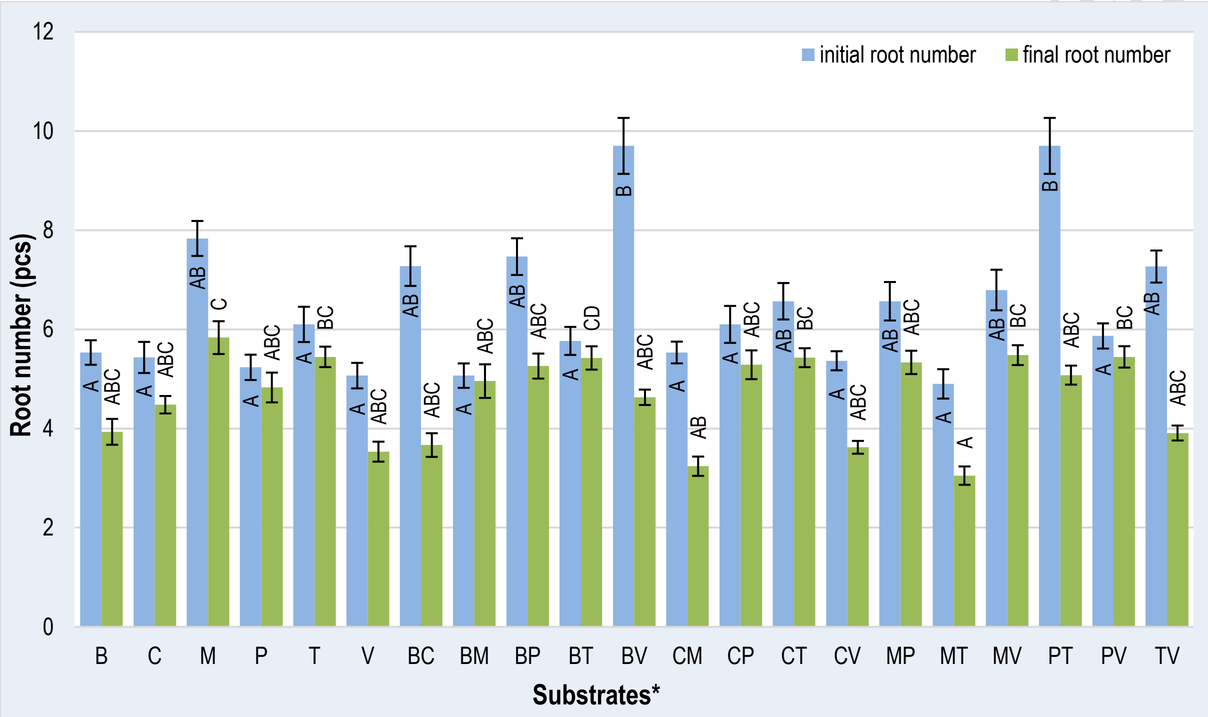

Figure 23. Root number means per substrates in the first acclimatization

In the case of the length of the longest root as the seventh morphological character (Figure 24), shown almost the same initial longest root averages for each substrate while the final values differed among them. The highest root averages were resulted by coco coir (39.40 mm) and coco coir + turf (41.71 mm), which were significantly higher than the others, even than the 3^{rd} and 4^{th} highest root length values on coco coir + perlite (31. 29 mm) and moss + turf (31.25 mm). At the other hands, the plants on bark substrate (8.07 mm) and bark + moss (10.91 mm) developed the shortest roots. In the case of the bark the longest root had a shorter final size than in the beginning of the acclimatization, showing a real difference ($p<0.05$)

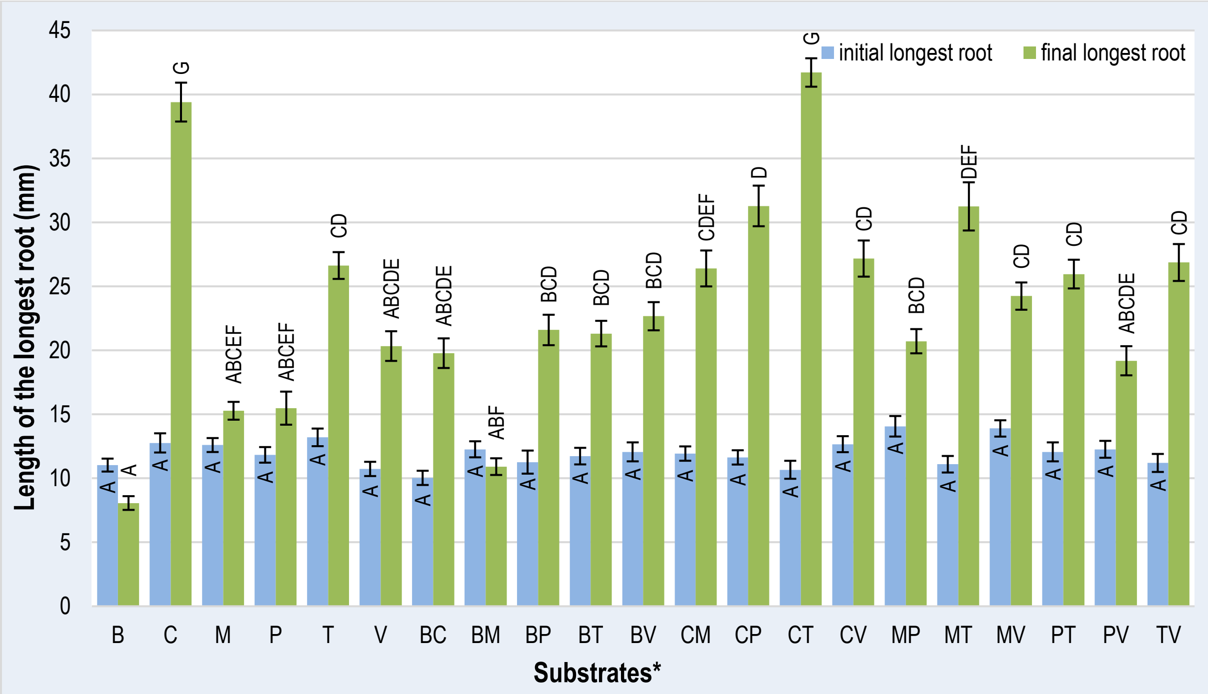

Figure 24. Longest root means per substrates in the first acclimatization

4.1.2. Physiological results of the first acclimatization

The chlorophyll contents presented in the Figure 25 shows an initial means with almost no significant difference among them with exception of the plants that were planted on bark (823 µg g-1), bark + vermiculite (554.2 µg g-1) and turf + vermiculite (596.26 µg g-1). After the acclimatization the highest content of chlorophyll were presented in bromeliads from moss (968.01 µg g-1), turf (987.4 µg g-1), coco coir + perlite (969.32 µg g-1) and moss + turf (1038.69 µg g-1). They did not show considerable variance among them, but compared to the smallest results of bark + vermiculite (372.67 µg g-1) and moss + perlite (532.22 µg g-1) the different was significant.

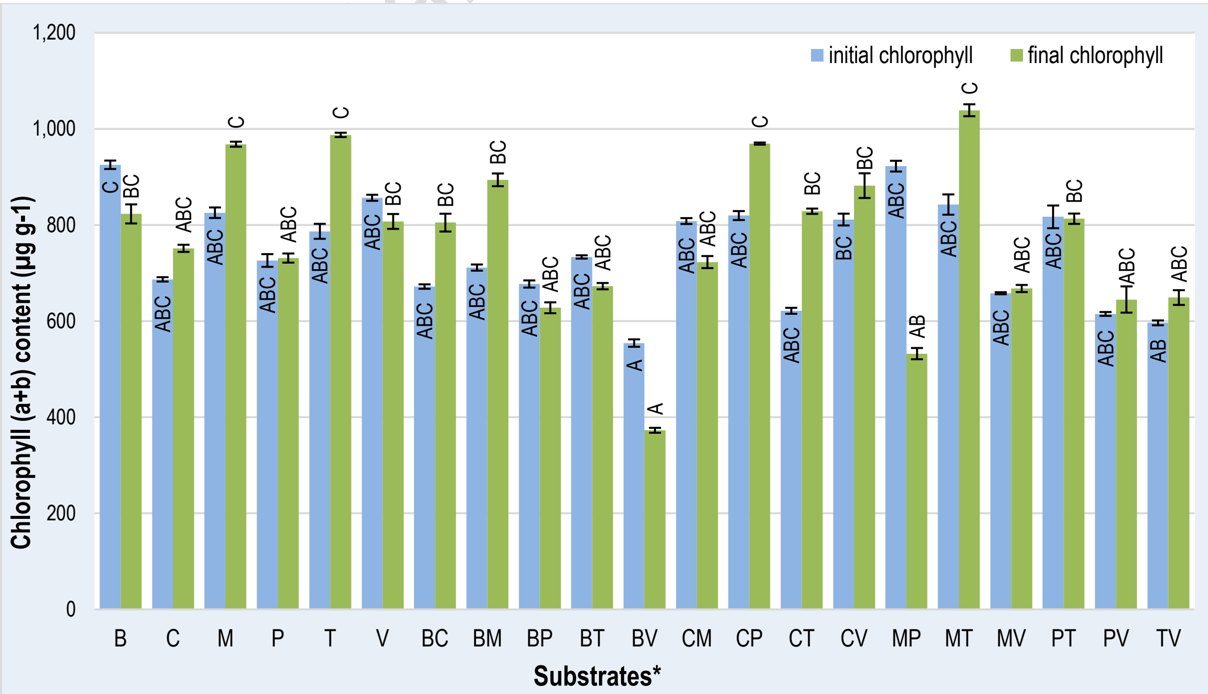

Figure 25. Chlorophyll content means per substrates in the first acclimatization

In the case of carotenoid contents (Figure 26), this variable decreased after acclimatization with exception of moss, turf, bark + moss, coco coir + turf, moss + turf. The plants that acclimatized on the latter substrate showed the highest value (21.82 µg g-1), and together with the closest means of turf (21.02 µg g-1), coco coir + perlite (21.41 µg g-1), moss (21.74 µg g-1), these results were significantly higher than the lowest carotenoid content identified in plants' leaves collected from bark + vermiculite (9.65 µg g-1) and moss + perlite (13.57 µg g-1).

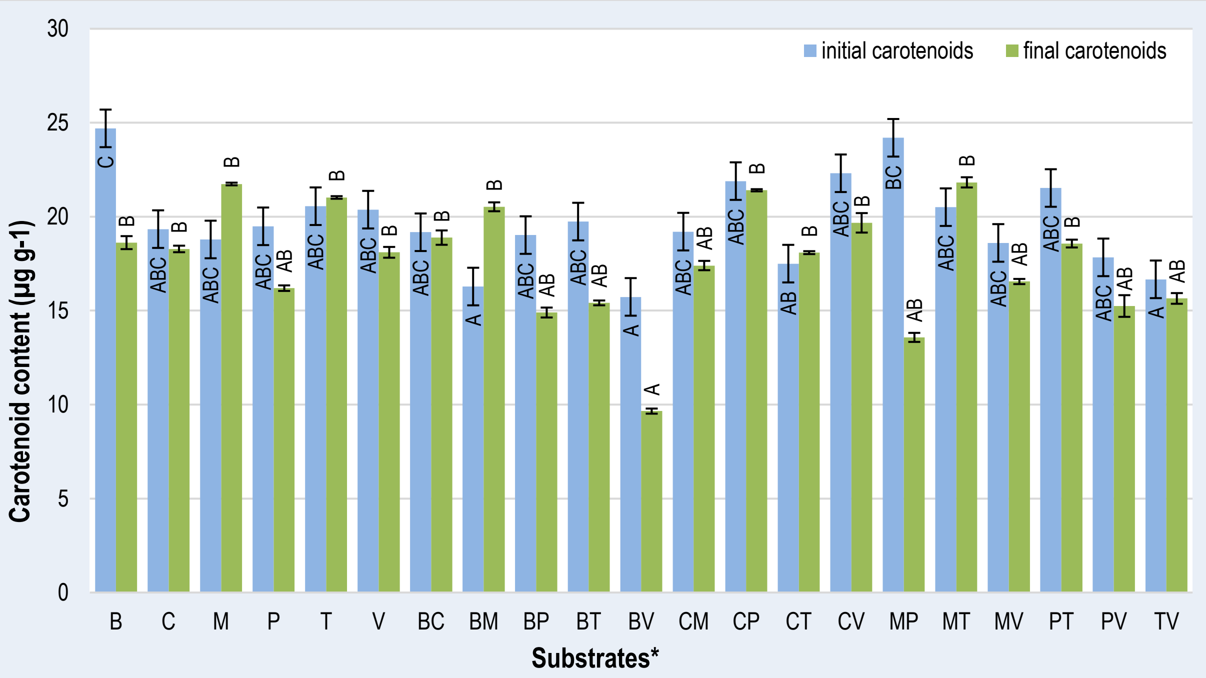

Figure 26. Carotenoid content means per substrates in the first acclimatization

4.1.3. Survival rate of the first acclimatization

In order to calculate which percentage of plants survived in each type of substrates, the survival rate of acclimatization was determined. The best substrate was vermiculate in which all plants survived, followed close by perlite, coco coir + vermiculite with the same value (96.67%) and coco coir + perlite, coco coir + turf with also equal ratio (93.33%). On the other hand, the bromeliads could not be able to acclimatized well on bark (50%), moss (60%) and moss + turf (66.67%), from which bark was the worst with 50 % of died individuals (Figure 27).

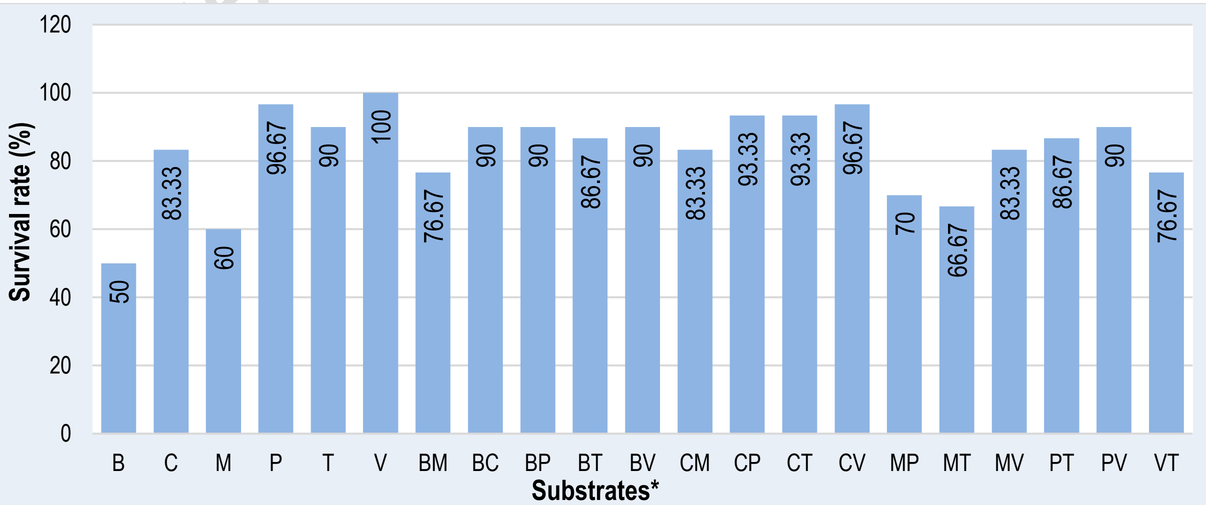

Figure 27. Survival rates of the first acclimatization experiment

4.2. Morphological and physiological results of the second acclimatization

4.2.1. Morphological results of the second acclimatization

According to the first morphological variable, shoot number showed no significance difference among the plants growing on different substrates at the initial stage and the same situation was repeated about the final shoot number of the plants at the end of the acclimatization. Most of the groups did not produce (or just little) more than 1.5 shoots (Figure 26)

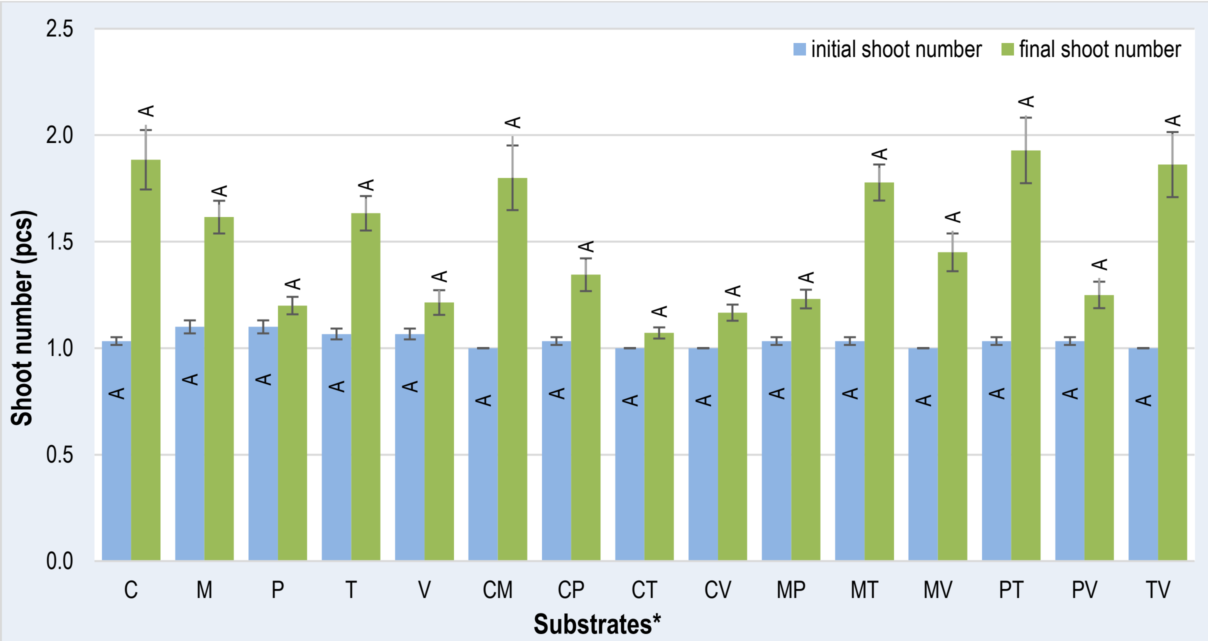

Figure 26. Shoot number means per substrates in the second acclimatization

The second physical character, plant height presented a notable increment at the end of the acclimatization (Figure 27). The highest plants were found on coco coir (67.96 mm), coco coir + vermiculite (67.6 mm) and coco coir + perlite (66.24 mm), and these values were significantly higher than almost all of the other stocks. On the other hand, the shortest plants (not significant different among them) were found on vermiculite (27.85 mm), perlite (27.2 mm) and as the lowest: perlite + vermiculite (27.08 mm). Additionally, from these substrates, the perlite resulted the lowest final height comparing the initial height (Figure 28).

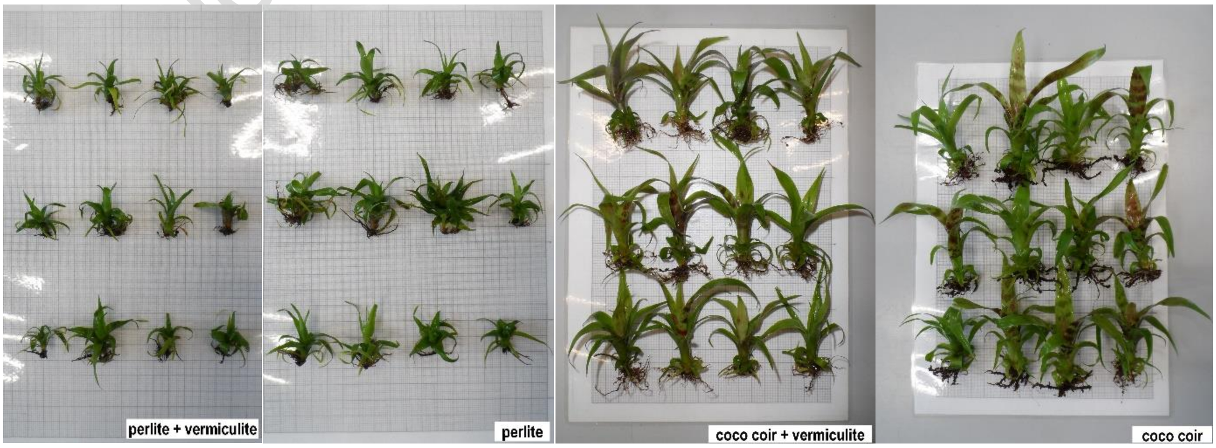

Figure 27. Perlite + vermiculite, perlite effected the lowest, coco coir + vermiculite, coco coir resulted the highest plants

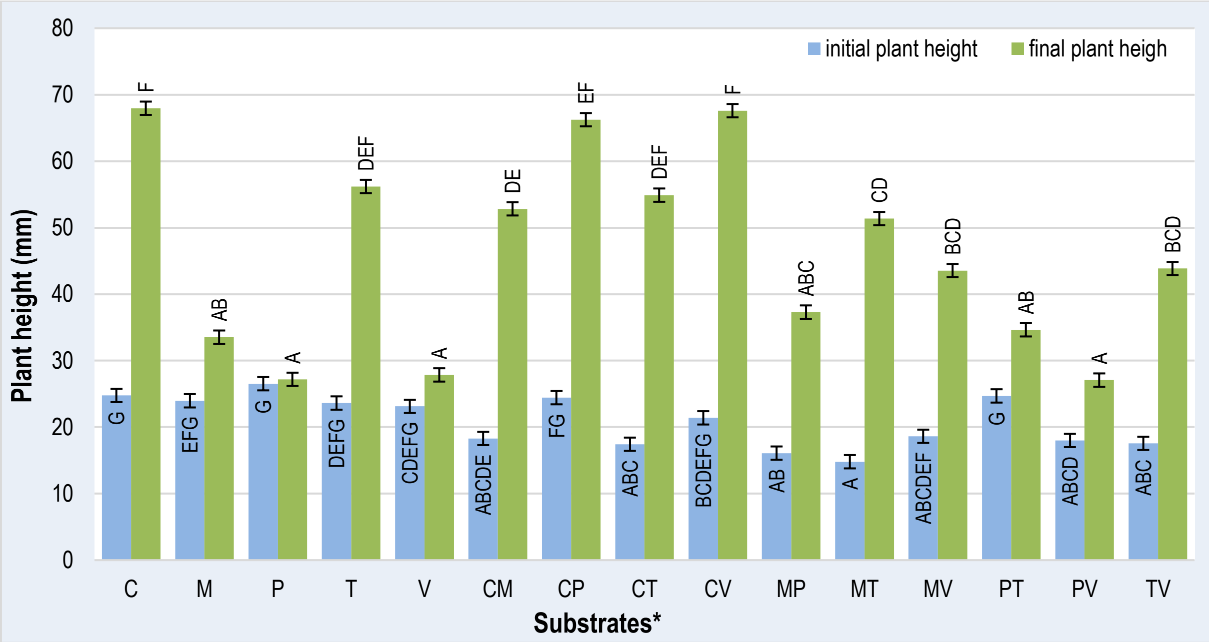

Figure 28. Plant height means per substrates in the second acclimatization

Belongs to the third morphological character, <u>plant weight</u> (Figure 29), there was not almost significant difference at the initial weight of the plants with exception of perlite (67.3 g) and coco coir + perlite mixture (33.73 g). At the end of the experiment (and compared with groups of turf, coco coir + perlite, coco coir + turf, moss + perlite, moss + vermiculite and perlite + turf), significantly the heaviest plants progressed on moss (186.3 g), turf + vermiculite (191.21 g), coco coir + vermiculite (192.6 g) and coco coir + moss (221.84 g). The lowest final weights were obtained on turf (46.08 g), coco coir + turf (53.6 g), and moss + perlite (53.14 g).

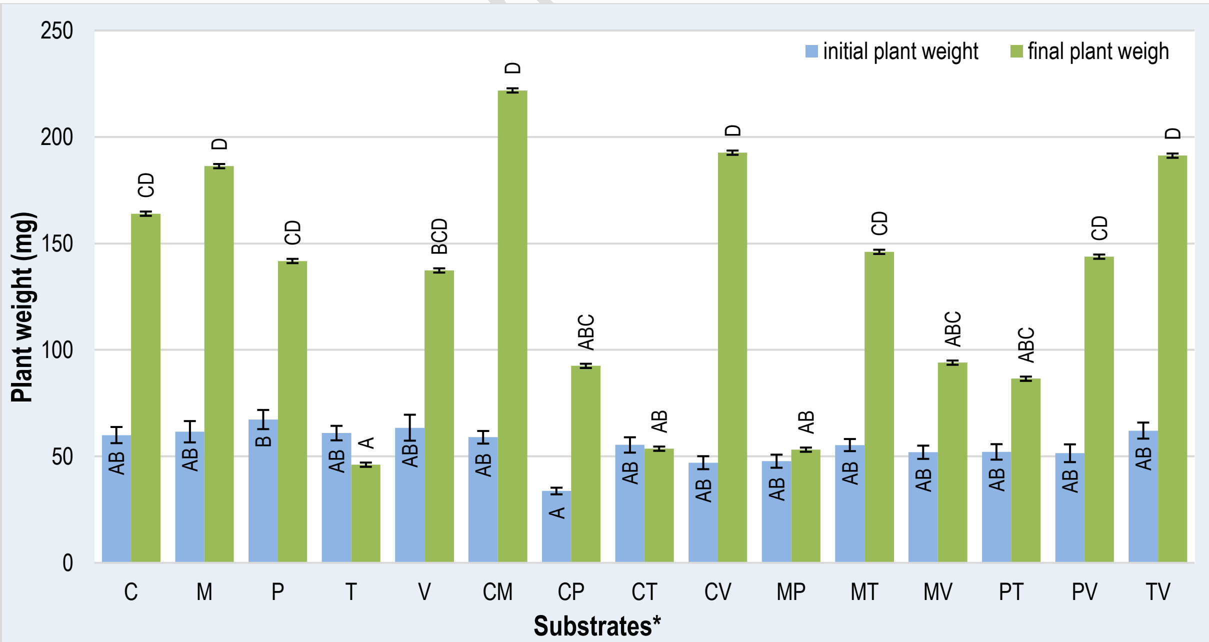

Figure 29. Plant weight means per substrates in the second acclimatization

Fourth, the <u>leaf length</u> (Figure 30) results showed almost non-different initial values, except perlite + turf (41.3 mm) and moss + turf (31.5 mm), which were the largest and smallest sizes, in order. In the case of the final sizes, the longest leaves were developed on coco coir (88.57 mm), coco coir + perlite (86.17 mm) and coco coir + vermiculite

(89.27 mm). Compared to almost all the other groups, these sizes were significantly higher, excepting the values of turf, coco coir + moss and coco coir + turf. The shortest leaves evolved on perlite (34.95 mm), vermiculite (32.42 mm), and perlite + vermiculite (33.42 mm). These were not significant different among them, but yes from the others.

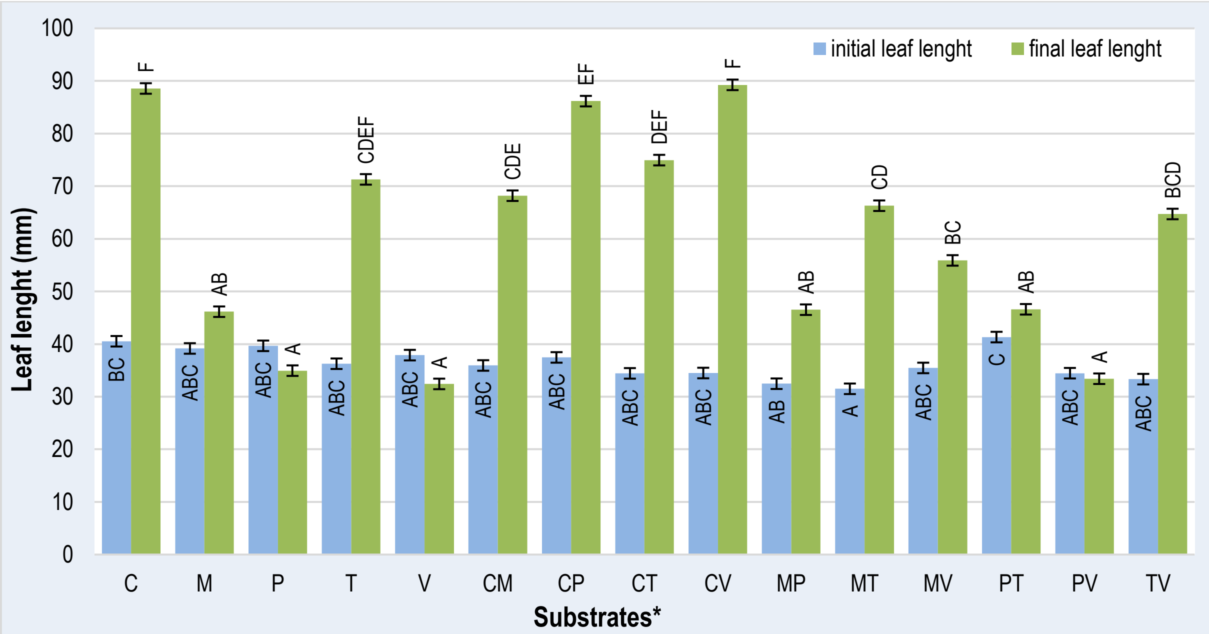

Figure 30. Leaf length means per substrates in the second acclimatization

The fifth morphological feature, leaf width (Figure 31), at first stage shows variable and overlapping error bars, showing not difference, except the particular case of perlite + turf (6.2 mm) and moss + turf (3.83 mm), being the biggest and shortest leaf in width. At the end of the acclimatization, the widest leaves appeared on coco coir (11.73 mm), coco coir + vermiculite (11.56 mm) and coco coir + perlite (10.14 mm). Moreover, the narrowest leaves were found in the cases of perlite + vermiculite (4.66 mm), vermiculite (4.57 mm) and perlite +turf (4.21 mm). To end, the unique significant difference was register in the plants with the shortest leaf width on perlite + vermiculate mixture (4.66 mm, $p < 0.05$).

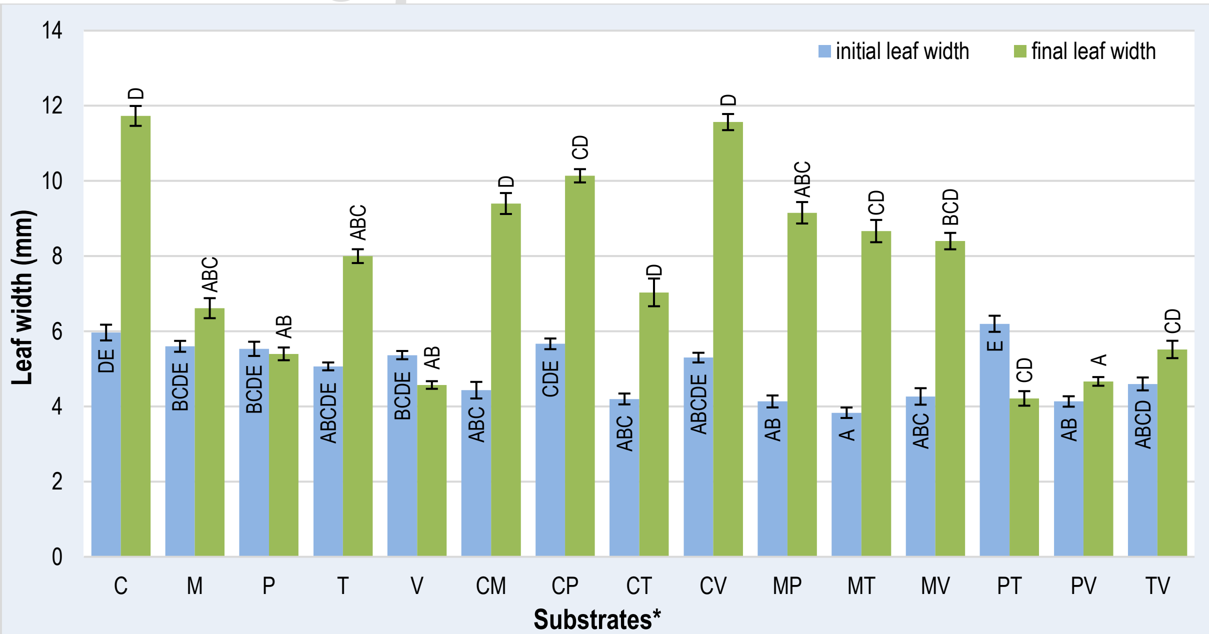

Figure 31. Leaf width means per substrates in the second acclimatization

According to the sixth character, the root number (Figure 32), which did not show significant differences at the beginning of the experiment, except for the particular case of coco coir + vermiculate (11.66 pcs) that resulted significantly more roots than almost the other substrates. In the case of the final values at the end of acclimatization, generally the number of roots decreased. The highest means belonged to the perlite + turf (7.33 pcs), vermiculite (6.9 pcs) and coco coir (6.86 pcs), but only perlite + turf resulted significantly the most roots compared with the lowest values on perlite + vermiculite (5.16 pcs), moss + perlite (5.1 pcs) and coco coir + moss (4.83 pcs).

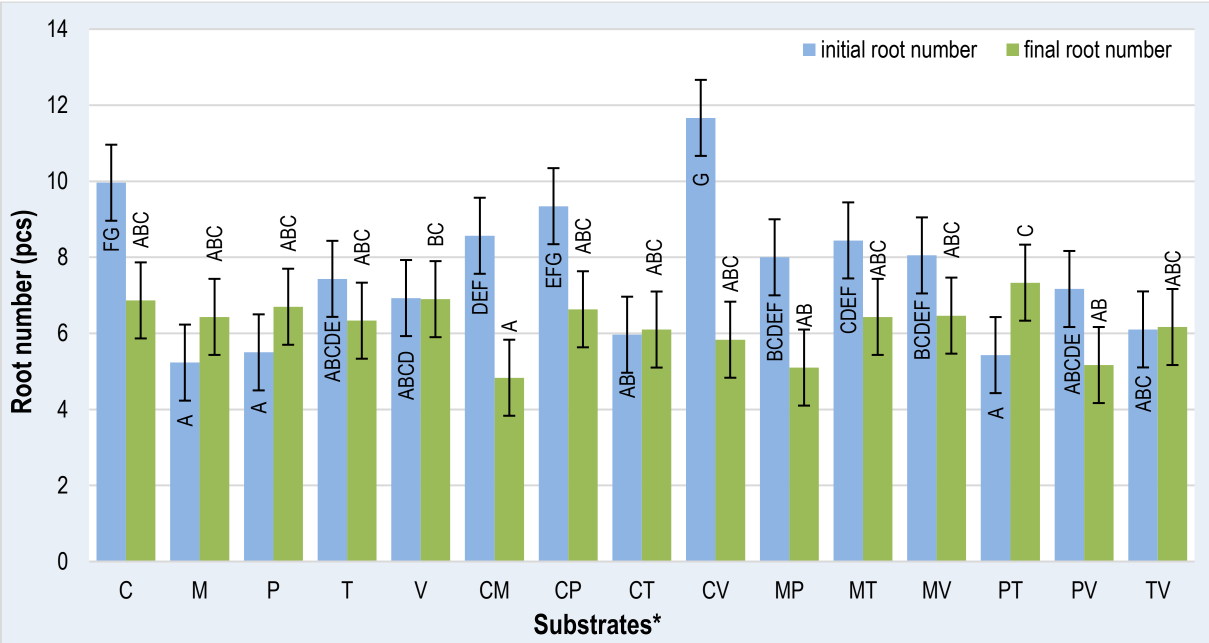

Figure 32. Root number means per substrates in the second acclimatization

Seventh, as the length of the longest root (Figure 32), at the initial stage the unique significant difference was detected between the groups of perlite (18.83 mm) and moss + turf (13.2 mm). However, at the acclimatization finalization, almost any substrate showed significant differences especially between groups with the highest/lowest values.

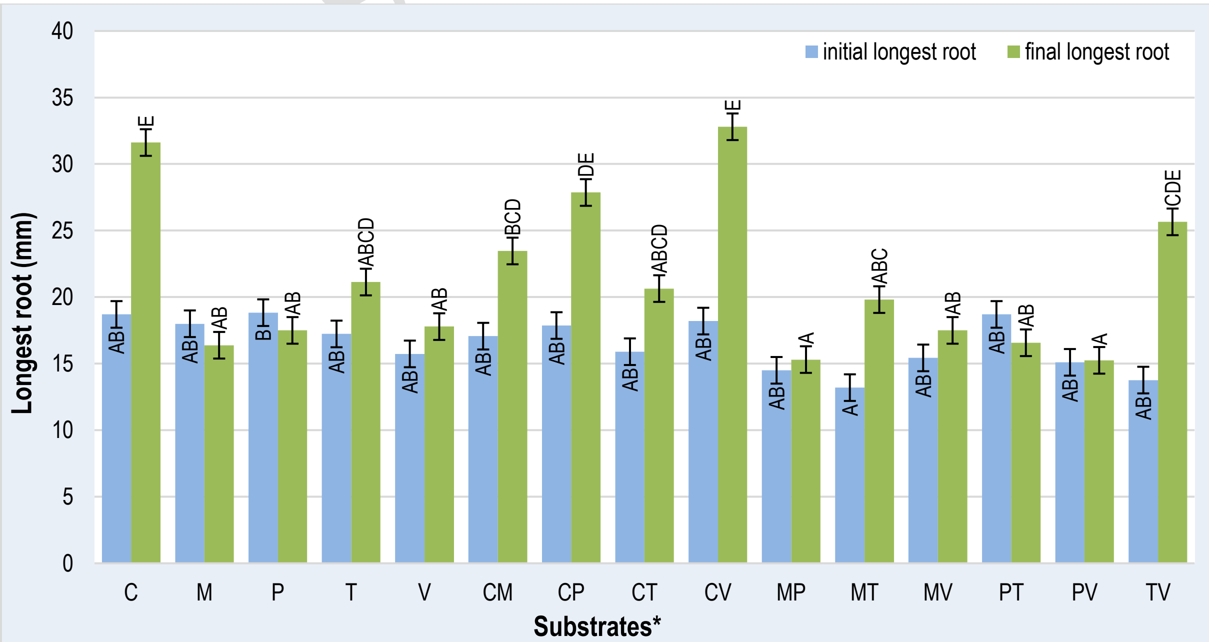

Figure 32. Longest root means per substrates in the second acclimatization

After this clarification, it is possible to mention the substrates on which the plants developed the longest root as: coco coir +vermiculite (32.8 mm), coco coir (31.62 mm), coco coir+ perlite (27.86 mm); and the shortest root as: moss (16.38 mm), moss + perlite (15.31 mm) and perlite + vermiculite (15.25 mm).

4.2.2. Physiological results of the second acclimatization

In general, the chlorophyll contents indicate an increment of values from the beginning and the end of acclimatization (Figure 33), and in the latter stage, the highest contents were detected on perlite (1068.05 µg g-1), coco coir + perlite (1046.68 µg g-1) and coco coir + vermiculite (1061.08 µg g-1), without significant differences among them. In the same way there is similarity among the means with the lowest chlorophyll levels in the cultivated plants originated from coco coir + moss (747.51 µg g-1), perlite + turf (723.59 µg g-1) and perlite + vermiculite (720.48 µg g-1), and these results already were significantly lower, compared with the highest ones.

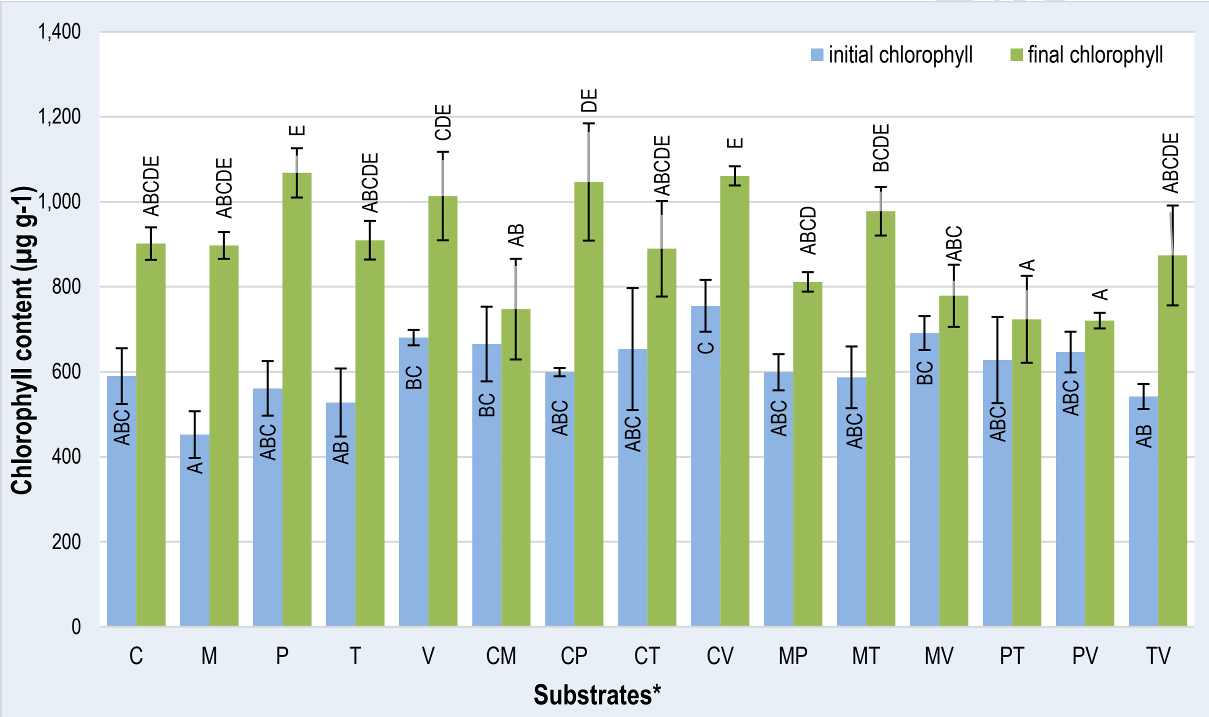

Figure 33. Chlorophyll content means per substrates in the second acclimatization

The carotenoid contents of the leaves (Figure 34) did not show too much difference between the beginning and the ending of the experiment. At the initial stage just moss substrate presented a significantly lower value (18.49 µg g-1) if compared to other groups with more than 24-25 different contend. At final stage the highest content of carotenoid were detected in the leaves of the bromeliads acclimatized on perlite (26.89 µg g-1), coco coir + perlite (26.52 µg g-1), coco coir + vermiculite (26.61 µg g-1), which were statistically similar. The lowest levels were received on coco coir + moss (19.35 µg g-1), perlite + vermiculite (19.6 µg g-1), perlite + turf (18.87 µg g-1).

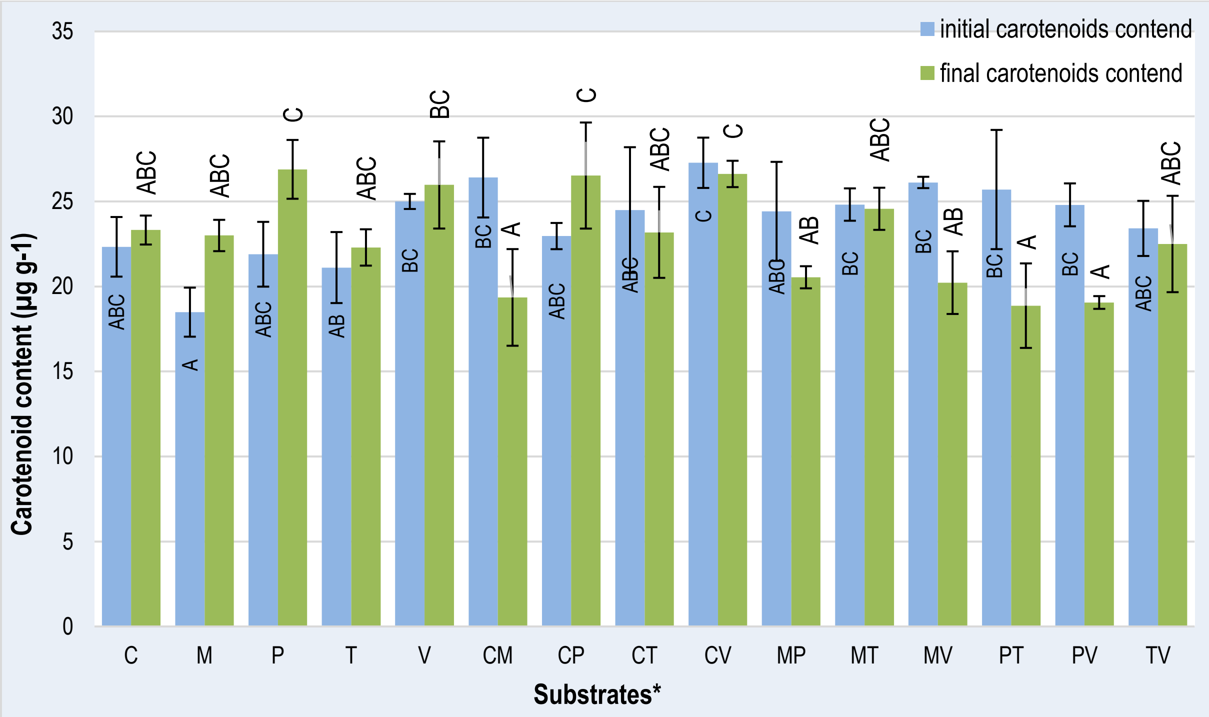

Figure 34. Carotenoid content means per substrates in the second acclimatization

4.2.3. Survival rate of the second acclimatization

The percentage of the survivor individuals ranged from 40 % to 100% (Figure 35). The highest ratio of individuals that survived total period were grown on turf, coco coir + moss, coco coir + vermiculite, with the same number of plants than at the beginning of the acclimatization. The lowest percentage was obtained in the case of the bromeliads that progressed on perlite + vermiculite mixture, with only 12 individuals when the experiment finished. Other not favorable substrates were moss (43.33 %), vermiculite (46.67%), and moss + perlite (43.33%).

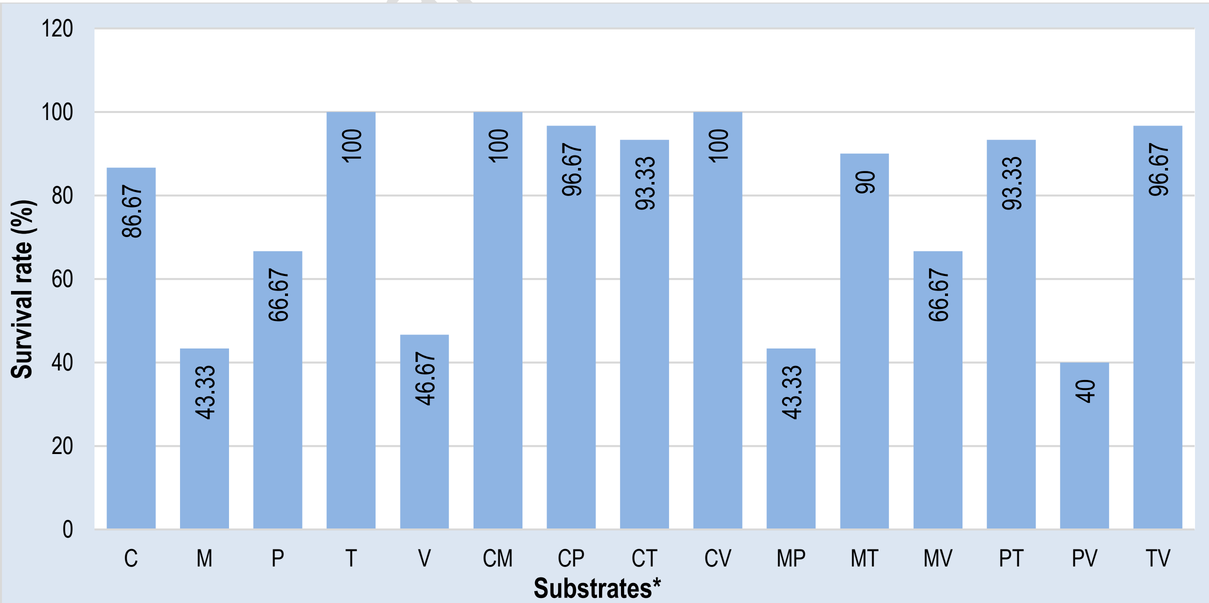

Figure 35. Survival rates in the second acclimatization

5. CONCLUSION

5.1. Morphological results

According to the shoot number, generally this character did not show any difference at the initial stage, which should be the ideal, but also at the final stage there was no significant difference in both (first and second) acclimatization, mostly. The special case of moss + perlite (resulted 1.57 shoots) during the first acclimatization could have been due to the presence of phytoregulators (Rech Filho et al., 2009), vitamins like B5 (da Silva et al., 2009), the age of the shoot - the juvenile tends to generate more shoots (Mercer & Kerbauy, 1992) or aggressive competitors (George et al., 2008) - the presence of moss together with perlite could be favor the growth of mosses instead of the bromeliads inducing a competition. The number of shoots could not be a good character to compare the acclimatization in the period of time of this study on the substrates.

Regarding to plant height, only in the first acclimatization one significant difference was recorded on bark + perlite with 51.4 mm. If perlite seems to decrease the mass aggregation (Gül et al., 2005). In other studies, bark stimulates the elongation, due to their low moisture, the plant elongates to look air humidity to absorb it by their leaves. For example, according with Martinez et al. (2020) *Tillandsia viridiflora* grew until 2 cm more than on a substrate with perlite + moss. Also, in other species as *Viburnum* spp. bark increases linearly the mass (Guérin et al., 2001).

In the case of plant weight, coco coir + vermiculite was the best in the first acclimatization (1.18 g). This mixture could provide nutrients and moisture to allow the plants increase their leaves number and the plant weight (Samori et al., 2016). In the second acclimatization, the heavier plants do not show a significant difference, but coco coir + vermiculite was among the best three. However, the lowest weight is the unique significant different value, turf (46.08 g). The turf or turf provide micropore structure, and also can wetting easily when it becomes dried (Sahin et al., 2002; Sahin et al., 2003). It could be providing the perfect media to allow the 100% acclimatization rate and as consequence, there is no stress to stimulate the leave growth or the water accumulation inside the leaves (Asaduzzaman, 2015).

Examining the leaf length during the first acclimatization, the significant different larger leaves developed on coco coir + turf (74.46 mm). The coco coir present longer leaves than turf, this suggests the biggest importance of coco coir on leaf growing providing nutrients and moisture (Samori et al., 2016) and the turf can help to keep this moisture and level of oxygen (Sahin et al., 2002; Sahin et al., 2003). In contrast, the smaller leaves were evolved on bark (37.67 mm), this result contradict the plant height result, which is compatible with the expectation of having a larger length (Martinez et al., 2020; Guérin et al., 2001). It could be explained thinking that the structure of bromeliads could be keeping the leaves erect, so it will be not necessarily having long leaves to have higher plants. During the second acclimatization, perlite (34.95 mm), vermiculite (32.42 mm), and perlite + vermiculite (33.42 mm), suggested that perlite and vermiculite do not favor the leaf length growth. One study suggests that perlite has a slow caption nutrient than others and less mass aggregation, influencing in the size (Gül et al., 2005). In the case of vermiculite, this result does not favor the previous studies that shows a superiority of vermiculite over other substrates in rooting and leaf formation (Navarro & Lopez-Perez, 2011). On the other hand, *Vriesea splendens* belongs to bromeliads where the leaves play an important role in the absorption nutrients and there is no too much studies to compare.

Belonging to the leaf width, in the first acclimatization, it was significant different for coco coir (6.88 mm), the contribution of this substrate in the nutrient and oxygen uptake and tissue formation (Samori et al., 2016; Sahin et al., 2002; Sahin et al., 2003) matches with the plant height and leaf sizes. Also, coco coir increases the water uptake and give K and P for leaf tissues (Ors & Anapali, 2010). On the other hand, bark had the lowest significant value in final leaf width (2.53 mm) which was smaller than the initial size; this result is influenced due to the low survival rate (50%) and low moisture retention capacity by bark. In the second acclimatization, the plants that raised on perlite + vermiculate mixture showed the shortest leaf width (4.66 mm). In warm conditions, perlite evaporates easy the water, obligating to reduce the leaf size in order to avoid evapotranspiration (Ors & Anapali, 2010). However, for some specific conditions like in tomato, perlite is efficient soilless substrate (Al-Shammari et al., 2018). Regarding to vermiculite as a mixture agent, it helps to increase the productivity and root or leaf sizes (Pisa et al., 2020), but this result cannot be expected if it is applied alone (Machi & Yamanouchi, 1993).

In the case of root number, during the first acclimatization, the plants developed more roots (5.8 pcs) on moss, but their length was short and this substrate showed only 60 % of survival rate. The moss in greenhouse conditions, where the amount of water was abundant than in the laboratory conditions (as first step of the second acclimatization), used their water retention capacity to grow (Sahin et al., 2002) and compete with the bromeliads for water. Also due to the structure of moss, it was too loose to have an easy attachment by bromeliads, so the plants tried to develop more and small roots to increase their anchorage (Smith, 1989). In the second acclimatization, significantly the fewest roots appeared on the mixture of coco coir + moss (4.83 pcs). In contrast of pure moss, the coco coir provides nutrition and hydration and not too loose substrate to reduce the need of root development. The other substrate that resulted few roots was the moss + vermiculite (6.46 pcs). On the other hand, this mixture presents the average of one of the longest root measures. These two results for moss + vermiculite could be affected by the low survival rate of 66.67% due to the competition and loose structure of moss, explained above. However, the vermiculite is a solid substrate that can lead the adhesion of the root, making it longest. This deduction according to the anatomy and morphological structure of *Vriesea,* where the roots are principal for anchorage and not for absorption (Silva et al., 2020).

In the first acclimatization, one of the longest root length averages was obtained on coco coir + perlite (20.71 mm). The coco coir allows a longest growth of the root because provide nutrients by N immobilization a strong C:N ratio, thanks to the microbiota (Holman et al., 2005). Also, the root can be influenced by the fact that perlite addition to coco coir increase the porosity and retain water time (Ilahi, & Ahmad, 2017). The average of shorter roots was presented on the plants from bark (8.07 mm). This result is explained due to the low survival rate and hydrophobic capacity of bark (Jerez, 2007). Some bromeliads in other studies developed also short and thin roots in *ex situ* conditions using bark (Alarcón & Rivera, 2007). For the second acclimatization it was not possible conclude because there was not any significant difference.

5.2. Physiological characters

The chlorophyll production changed and increased during acclimatization. This physiological character is an indicator of the metabolism, chloroplast and photosynthetic activity. Considering the highest chlorophyll averages from moss (968.01 µg g-1), turf (987.4 µg g-1), coco coir + perlite (969.32 µg g-1) and moss + turf (1038.69 µg g-1) in the first acclimatization and during the second trial, perlite (1068.05 µg g-1), coco coir + perlite (1046.68 µg g-1) and coco coir + vermiculite (1061.08 µg g-1). In both cases coco coir + perlite mixture had plants with high chlorophyll accumulation. The nutrient content from coco coir and the porosity from perlite made this mixture a good combination for healthy plant production and developing darker leaves according to other studies on strawberries (Wortman et al., 2016; Bidarnamani, 2023), *Lilium* (Nikrazm et al., 2011), sunflower and corn (Holman et al., 2005). In the first acclimatization, bark + vermiculite (372.67 µg g-1) and in the second experimentation, perlite + vermiculite (720.48 µg g-1) resulted the lowest chlorophyll concentrations. Although these values suggest that, the vermiculite is related with low chlorophyll accumulation, and as a media to transport nutritive substances (Shi & Byrne, 1995). In other studies, vermiculite in soilless systems as porous tube-vermiculite had increased the photosynthetic conditions due to high enzymatic activity and efficiency in nutrient conductance (Wang et al., 2019). Also, vermiculite can improve potting mixtures and be a good media to transmit iron, potassium and calcium to the plants (Mozafar & Oertli, 1988).

Regarding to the carotenoid content, in the second acclimatization, it is difficult to observe clear results because there are no significant differences. In the first acclimatization, it is possible analyses the role of turf with a well carotenoid accumulation alone and in combination with moss. During the second acclimatization, seems that the coco coir mixtures produce a relatively high content of carotenoid, except when it is mixed with moss. It is expected than in laboratory conditions (as first step of the second acclimatization) the carotenoid production will be lower than in greenhouse, due to the low but constant light intensity (Fuentes et al., 2006). In the case of the temperature, the formation of carotenoids increases in lower temperatures as an acclimatization resource (Pedroso et al., 2010), as the conditions were constant the carotenoids did not change too much from the initial stage until the end of the experiments. Nutrients as magnesium and nitrogen can increase the production of carotenoids (dos Santos et al., 2019). Moreover, the carotenoids depend also from the species this can change among the same genus in bromeliads (Tamaki et al., 2011). For these reasons more repetitions or longer time for experimentation, maybe will provide significant difference for carotenoids.

5.3. Acclimatization (survival) rate

The influence of substrates for all the morphological and physiological characters of *Vriesea splendens* individuals described in this study influenced on the survival rate.

In the first acclimatization, only on vermiculite all the initial individuals survived. The vermiculite in this first trial conditions had a regular performance in all the measured characters. According to the literature, vermiculate due to their water holding capacity can improve the chlorophyll, root and leaves production (Pisa et al., 2020), showing in this study a surprised higher survival rate than the coco coir substrate. In contrast in the second acclimatization, vermiculite (46.67.%) was one of the three worst survival rate, it can be explained by the small size in plant height, leaf length, few roots, short root and low chlorophyll accumulation presented by plants planted on vermiculite. One explanation for this performance is the low ventilation in the first part of the second acclimatization (in laboratory conditions) allowed that the vermiculite stays wet longer, which due to the natural habitat of *Vriesea* should improve their growth. Other factor that could affect is the light intensity, which can decrease the productivity on bromeliads (Martins et al., 2015; Medina et al., 1986).

The favorite in many studies is for sure coco coir due to their moisture, nutrient content, and free diseases substrate. This study is not the exception, coco coir in three different combinations showed a high acclimatization rate in mixtures in the first acclimatization with perlite (93.33%), turf (93.33%) and vermiculite (96.67%) and in the second trial favored the plants on coco coir + vermiculite (100%) and coco coir + moss (100%). Coco coir increased the plant mass in the size and number of leaves (Sasamori et al., 2016), because it provides oxygen and easy nutrient access as carbon and nitrogen (Holman et al., 2005; Sahin et al., 2002; Sahin et al., 2003). In addition, the plants could develop long roots by the porosity and moisture retention (Ilahi, & Ahmad, 2017).

The turf in the first acclimatization had an unexpected performance in combination with moss of survival rate (66.67 %). However, plants developed longer leaves together with coco coir. On the other hand, in the second acclimatization showed the lowest plant weight contrasting the expected water contend or tissue production in the plant (Asaduzzaman, 2015). It should be due to the no stress conditions because turf had 100% acclimatization rate. It could suggest that under controlled conditions in temperature and light, turf is excellent substrate for acclimatization, even though the plants will not be big as in the case of coco coir.

Another dual substrate (in the first acclimatization) was the moss as pure medium (60%) and in combination with turf (66.67%) showed the lowest survival rates. Moreover, during the second acclimatization, moss also showed low survival rate alone (43.33 %) and in combination with perlite (43.33%). The low shoot numbers, low survival rate and observations along the experiment suggest that the mosses started to compete with bromeliads. While bromeliads try to develop many short roots to get the appropriate anchorage, mosses growth faster (Smith, 1989). It reduces the survival rate but increase the root number.

The worst acclimatization rate had the plants on bark with only 50%. The plants that acclimatized on bark presented very short roots, which is common in *ex situ* conditions (Alarcón & Rivera, 2007). Also, the size of the bark could have been too big leaving a lot of space to be reached by the tiny initial roots, reducing the nutrient absorption from the substrate. Another observation is that the pine bark could be too hard to be penetrated by the plant root, defaulting the leaves of the water tank position to the reception of water and organic matter decomposition (Vanhoutte et al., 2017). For this reason, bark was not used during the second acclimatization.

In conclusion, there is few physiological and morphological research that focus only on *Vriesea splendens*, despite its economic importance. However, useful information can be obtained from closely related species of the same genus or *Tillandsioideae* subfamily.

In most cases, the physiological characters did not show a significant difference among the substrates, so increase the time of experimentation could be an alternative in future experiments to look for a better comprehension of the role of chlorophyll and carotenoids in acclimatization process.

Regarding to the best substrates in both first and second acclimatization, coco coir could be the most adequate option showing, not always the best, good averages in plant size and weight, leave size, root size, root number and survival rate. Other substrates that can be recommended to be used in the first experiment (acclimatization in one step, in greenhouse) is vermiculite with the maximum survival rate, and in the second trial (acclimatization in two steps: at first - in laboratory, and after - in greenhouse), turf also presented all the individuals alive at the end of the experiment.

Besides that, most of bromeliads and also *Vriesea splendens* 'Fire' are epiphytes in trees, but their acclimatization on a pure bark substrate is not recommended. In addition, according with these results, the moss is not suggested, besides it is a proper element in the natural habitat of bromeliads, they reduce the performance of bromeliads taking advantage of moisture conditions.

More studies are needed to understand not only the morphology or physiology but also the genetic response in the acclimatization rate. These experiments not only contribute for the flower industry, but also for the ex-situ conservation and bromeliad's natural habitat protection.

7. SUMMARY

Two independent experiments were developed to test the acclimatization process of *Vriesea splendens* 'Fire' on different substrates, such as, bark (B), coco coir (C), moss (M), perlite (P), turf or turf (T), vermiculite (V), bark + coco coir (BC), bark + moss (BM), bark + perlite (BP), bark + turf (BT), bark + vermiculite (BV), coco coir + moss (CM), coco coir + turf (CT), coco coir + vermiculite (CV), moss + perlite (MP), moss+ turf (MT), perlite + turf (PT), perlite + vermiculite (PV), turf + vermiculite (TV). The characters evaluated were morphological (number of shoots, plant height, plant weight, leaf long, leaf width, root number, root length) and physiological (chlorophyll and carotenoid content).

The shoot number was higher on moss + perlite (1.57 pcs) in the first acclimatization. The plant height was affected in a positive way by the bark + perlite mixture (51.4 mm) also in the first trial. Belong to plant weight coco coir + vermiculite showed a significant increment in the individual's weight (1.18 g) in the first, and turf demonstrated the lowest plant weight (46.08 g) in the second experiment. In leaf length character only in the first trial, coco coir + turf had the superior (74.46 mm) and bark substrate had the lowest (37.67 mm) average. Regarding to leaf width, the widest leaves were developed on coco coir (6.88 mm) and the narrowest on bark (2.53 mm) in the first trial, and the less wide leaves from perlite + vermiculate mixture (4.66 mm) in the second acclimatization. In the case of the root number, the plants of the first trial produced more roots in moss (5.8 pcs) than the others, and in the second experiment coco coir + moss (4.83 pcs) and moss + vermiculite (6.46 pcs) showed less root number. The longest root only in the first acclimatization showed significant results presenting the largest root average on coco coir + perlite (20.71 mm) and the shortest mean on bark (8.07 mm).

Mostly, the physiological characters did not show a significant difference among the substrates, so increase the time of experimentation could be an alternative in future experiments to look for a possible concrete result. Finally, the acclimatization rate was also evaluated. It was the best with the 100% of individuals alive on vermiculite (in the first experiment) and turf, coco coir + moss, coco coir + vermiculite (in the second acclimatization). In the latter trial, bark resulted the lowest survival rate (50%), and in the first study, pure moss, moss + perlite equally effected the lowest value (not more than 44%).

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10. APPENDIX

Table 1. Summary of *Vriesea* genus in vitro and acclimatization studies

Authors	Species	Substrate used in acclimatization	Treatments applied	Survival rate	Main results
Ördögh, M., 2022	*Vriesea splendens* 'Fire'	Mixed substrate: vermiculate + peat + perlite (1:1:1)	In vitro at 22-25 °C for 3 months on MS culture medium with 0.1-0.8 mg L-1 BAP, BAPR, KIN, MT, NAA, IBA	70 % for KIN, IBA, NAA treatments	Hormones for root stimulation are important to acclimatization
Pedroso et al., 2010	*Vriesea inflata (Wawra) Wawra*	commercial substrate: fine Pinus bark sterilized	In vitro at 15 °C for two years and transferred to in vitro conditions for 3 months: 1) at 15 °C 2) at 28 °C	100% for all treatments	Leave length at 15 °C were smaller than those at 28 °C.
Alves et al., 2006	*Vriesea reitzii*	Composed substrate: 1:1 (v/v) of carbonized rice coat and Turfa Fertil1 mineral supplement.	Nodular culture induction: MS culture medium supplemented with NAA (2.0 mM) and BAP (4.0 mM)	90 % for plantlets longer than 3 cm	Basal leaves as explants showed 90.6% induction rate of nodule clusters in MS culture medium supplemented with 20.0 mM 2,4-D and 1.0 mM KIN
de Resende et al, 2016	*Vriesea cacuminis*	Comercial substrate: Plantmax Hortaliças HT® (Eucatex Agro, SP)	In vitro cultures on MS medium with: BA (0, 5, 10 or 15 µM), GA3 (0, 5, 10 or 15 µM), NAA (0, 1.5, 3 or 4.5 µM)	95% for plantlets from culture media GA3 and 85% for plantlets from BA	Highest microcutting rooting was found as response to the medium supplemented with NAA at 0.2 µM
Dal Vesco et al., 2014	*Vriesea reitzii*	Composed substrate: mixture of carbonized rice coat, pine bark and mixture of commercial Plantmax® (2:2:1 v/v)	Nodular culture induction: MS basic (MSB), liquid or gelled, supplemented or not with NAA, BAP or TDZ	95% was the highest for plantlets in liquid MSB medium with GA3 (10 µM)	Induced nodular culture had MSB medium with NAA (4 µM) and sub-cultivated on MSB medium with NAA (2 µM) plus 2-iP (2 µM) showed granular texture and high proliferation rate
Rech Filho et al., 2009	*Vriesea reitzii*	Composed substrate: 1:1 (v:v) of carbonized rice coat and Turfa Fertil® mineral supplement (N-4, P2 O5 - 14, K2 O-8)	Nodular culture induction: 1. MS free of plant growth regulators 2. MS + NAA (2µM) and BAP (4µM), 3. MS + GA3 (10µM).	100% in all treatments	The subculture to MS liquid medium plus GA3 (10µM) and in MS liquid medium free of plant growth regulators resulted in a high proliferation rate
Rech Filho et al., 2005	*Vriesea reitzii*	Composed susbstrate: 1:1 (v:v) of carbonized rice coat and Turfa Fertil mineral supplement (N-4, P2O5-14, K2O-8)	In vitro MS and LPm liquid media, supplemented with: 1. BA 2. NAA 3. GA3	100% on shoots longer than 2 cm	Shoot elongation was observed in MS medium supplemented with GA3. MS medium supplemented with NAA and BA helped on proliferation system

Freitas et al., 2015	*Vriesea inlata (Wawra) Wawra*	Commercial substrate: Pinus bark at 100%	In vitro in basal media: 1. without carbohydrates 2. media containing 1.0; 1.5; 3.0; 4.5; and 6.0% (w/v) of sucrose	100% for all treatments	1.7% sucrose for 60 days promotes root growth, which leads to more vigorous plants in acclimatization.
Kämpf et al., 2009	*Vriesea philippocoburgii*	Composed substrate: 1:2 (v:v) coconut powder and broken expanded clay	Elemental sulfur (0, 0.5, 1, 2 and 4 g sulfur/L substrate)	100% for all treatments but difference in tissue damage	0.5 g S/L substrate provided the best results
Sasamori et al., 2018	*Vriesea incurvata*	Commercial substrate: Carolina Soil®, with a base of peat, vermiculite, and carbonized rice hulls	Influence of sucrose (10, 30 and 60 g L-1)	100% for all treatments	Sucrose at 60 g L-1 provided highest length of the aerial portion and of the longest root, greatest number of leaves and roots, highest fresh mass and lowest contents of chlorophylls and carotenoids. Favor acclimatization
Sasamori et al., 2019	*Vriesea flammea*	Commercial substrate: sphagnum and crushed stone	MS medium: 1. 25 and 50% of nitrogenous salts and macronutrients) 2. Different concentrations of sucrose (20, 30, 40, 50 and 60 g L-1)	76% survival with low concentrations of nitrogenous salts and macronutrients	The highest sucrose concentration promoted aerial system and fresh mass of acclimatized plants

DECLARATION

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