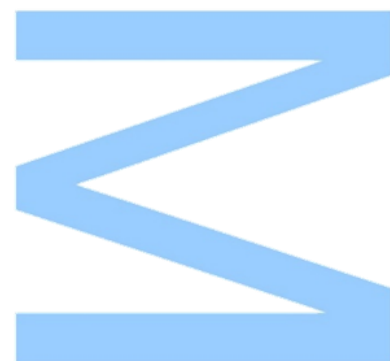
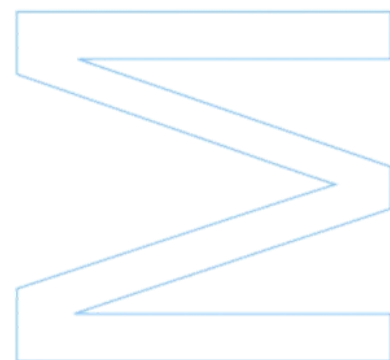


Impact of near-UV light on several *Catharanthus roseus* cultivars: a morpho- physiological analysis

João Emanuel da Costa Prada

Estágio curricular de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto
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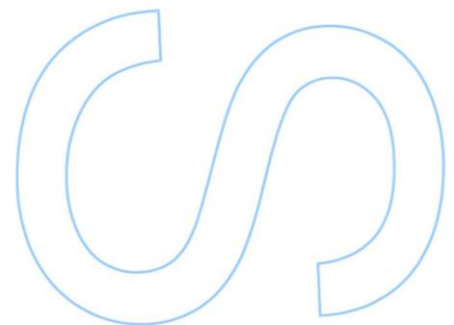
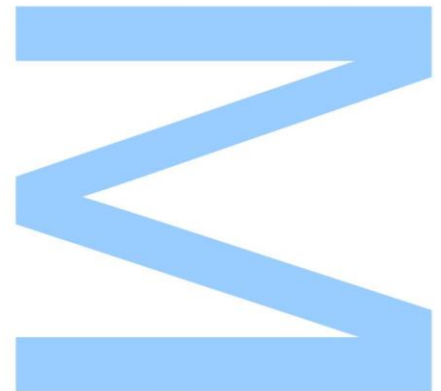
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Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ____/____/____

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Abstract

The production of anti-neoplastic alkaloids is, nowadays, of extreme importance and value, due to the increasing incidence of cancer on human population, which is projected to continue to rise. The diminished natural availability of Vinblastine and Vincristine, forces the need to synthesize these molecules in laboratory, which is highly costly. Alkaloid production by *C. roseus* is still not satisfactory to supply the crescent demand that the pharmaceutical companies need. *C. roseus*' alkaloid production needs to be enhanced, in order to supply the pharmaceutical market with much higher quantities of these valuable alkaloids, with less expensive methodologies.

N-UV light-spectrums are reported to manipulate several morphological, physiological and metabolic parameters. These light-spectrums increase leaves' dry weights and thickness, induce flowering and are of crucial importance to enhance Vinblastine and Vincristine production in *C. roseus*, by promoting Catharanthine oxidation. This experimental work aims to understand the impacts that N-UV light spectrum has on *C. roseus*' growth and development, and to perform a screening with 9 *C. roseus* cultivars (Titan Polka Dot, Cora Deep Lavender, Cora Burgundy, Sunstorm Deep Lilac, Cora Red, Cora XDR Punch, Polka Dot Apricot, Cora XDR White and Sunstorm Apricot) with the objective of pinpoint one or more cultivars that reveal to be, morpho-physiologically, more prone to be utilized in industrialized alkaloid production.

Results indicate that Titan Polka Dot, Cora Deep Lavender, Polka Dot Apricot and Cora XDR White are the more prone cultivars, in their morpho-physiological parameters, to be industrialized towards massive alkaloid production. Most of these cultivars had significantly higher results in plant height, number of leaves, leaf area, total dry weight, leaves dry weight, flowers dry weight and number of produced flowers and flowering time, in comparison to Sunstorm Deep Lilac and Sunstorm Apricot. These results are exclusively dictated by the cultivars' genetic characteristics. However, Polka Dot Apricot and Cora XDR White shown to have a significantly lower photosynthetic rate, in comparison to Sunstorm Deep Lilac and Sunstorm Apricot.

The N-UV light treatment applied to plants in this experiment did not exhibit any statistically significant results, with the exception in the number of leaves. These results indicate that the N-UV light treatment applied did not stress *C. roseus* plants in analysis, neither enhanced their morpho-physiological production.

Titan Polka Dot, Cora Deep Lavender, Polka Dot Apricot and Cora XDR White are, therefore, the most interesting cultivars to be used in industrialized alkaloid production, concerning their morpho-physiological production. However, their alkaloid content

should be tested previously. Moreover, N-UV light treatment applied must be perfected, with the aim of enhancing the morpho-physiological parameters of *C. roseus*.

Keywords: *Catharanthus roseus*; Morpho-physiology; Screening; Vinblastine; Vincristine;

Resumo

A produção de alcaloides anti-neoplásicos é, hoje em dia, de uma importância e valor enormes, devido ao contínuo aumento da incidência de cancro na população humana, incidência que se projeta continuar a aumentar. A diminuta disponibilidade natural de Vimblastina e Vincristina obriga a produção artificial destas moléculas em laboratório, procedimento que é muito caro. Por outro lado, a produção destes alcaloides pelo *C. roseus* não é, ainda, satisfatória para fornecer ao mercado farmacêutico a quantidade exigida pela indústria, pelo que esta produção deve ser estimulada e otimizada, possibilitando assim o fornecimento da indústria farmacêutica com as quantidades desejadas, recorrendo a métodos de produção mais económicos.

Os espectros de luz N-UV são capazes de manipular vários parâmetros das plantas, nomeadamente morfológicos, fisiológicos e metabólicos. Este tipo de espectros de luz aumentam o peso seco de folhas, bem como a sua espessura, participam no processo de floração, e são importantes na síntese de alcaloides Vimblastina e Vincristina, por promoverem a oxidação de Catarantina.

Esta investigação experimental tem como objetivo entender o impacto do N-UV no crescimento e desenvolvimento de *C. roseus*, e também realizar um 'screening' com 9 cultivares de *C. roseus* (Titan Polka Dot, Cora Deep Lavender, Cora Burgundy, Sunstorm Deep Lilac, Cora Red, Cora XDR Punch, Polka Dot Apricot, Cora XDR White e Sunstorm Apricot), com o objetivo de declarar uma ou várias cultivares que se destaquem na sua adequabilidade, do ponto de vista morfo-fisiológico, a serem utilizadas numa produção industrial de alcaloides.

Os resultados obtidos mostram que as cultivares Titan Polka Dot, Cora Deep Lavender, Polka Dot Apricot e Cora XDR White são as mais adequadas (morfo-fisiologicamente) para serem escolhidas numa produção industrial, por se destacarem com resultados significativamente superiores em parâmetros como a altura da planta, número de folhas, área foliar, peso seco total, peso seco das folhas, peso seco das flores, número de flores produzidas e tempo até floração, quando comparadas com as cultivares Sunstorm Deep Lilac e Sunstorm Apricot. Estes resultados derivam dos recursos genéticos de cada cultivar. No entanto, Polka Dot Apricot e Cora XDR White obtiveram resultados significativamente inferiores a Sunstorm Deep Lilac e Sunstorm Apricot em alguns dos parâmetros fisiológicos, nomeadamente a taxa fotossintética e a taxa de trocas gasosas.

O espectro de luz N-UV fornecido às plantas durante a experiência não provocou resultados significativos de relevo, com exceção no parâmetro 'número de folhas'. Estes resultados indicam que o espectro de luz N-UV aplicado não teve efeitos negativos nas

plantas, pela verificada ausência de sinais de stress. Por outro lado, também não foi suficiente para melhorar a produção, do ponto de vista morfo-fisiológico, devido à fraca intensidade da luz aplicada.

Titan Polka Dot, Cora Deep Lavender, Polka Dot Apricot e Cora XDR White revelam-se, então, as cultivares mais interessantes para serem utilizadas numa produção industrial de alcaloides, no que toca aos seus parâmetros morfo-fisiológicos. No entanto, a sua produção de alcaloides deve ser testada previamente. Mais, o espetro de luz N-UV fornecido às plantas tem de ser aperfeiçoado e o seu impacto maximizado, com o objetivo de melhorar os parâmetros morfo-fisiológicos de *C. roseus*.

Palavras-chave: *Catharanthus roseus*; Morfo-fisiologia; Screening; Vimblastina; Vincristina;

Abbreviations

APR – Sunstorm Apricot
B-light – Blue light spectrum
BUR – Cora Burgundy
CAT – Catharanthine
CDL – Cora Deep Lavender
CIMg – Magnesium chloride
DAT - 4-O-deacetylvindoline 4-O-acetyltransferase
DM – Dry matter
FMN – Flavin mononucleotide
FR-light – Far-red light spectrum
IPP – Isopentenyl diphosphate
MAP – Medicinal and Aromatic Plant
MIA – Monomeric Indole Alkaloid
N-UV – Near-ultraviolet light spectrum
PAC – Pacífica
PAR – Photosynthetic active radiation
PDA – Cora XDR Polka Dot Apricot
Pr – Phytochrome red
Pfr – Phytochrome far red
PUN – Cora XDR Punch
R-light – Red light spectrum
RB – Red and Blue light spectrums
RED – Cora Red
RS Ratio – Root/Shoot Ratio
SDL – Sunstorm Deep Lilac
SLS – Secologanin synthase
SRH – Sunstorm Red Halo
TIA -Terpenoid Indole Alkaloid
TIT – Titan Polka Dot
UV - Ultraviolet
VBL – Vinblastine
VCR – Vincristine
VDL – Vindoline
WHI – Cora XDR White

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1. Introduction

1.1 *Catharanthus roseus*

1.1.1 *Taxonomical and morphological approach*

Catharanthus roseus, most known as Vinca or Madagascar periwinkle, is an herbaceous perennial plant, native to the Madagascar island. This angiosperm is a dicotyledon, belongs to the Apocynaceae family and to the genus *Catharanthus*, and it has been produced as an ornamental plant in several world regions. This plant is considered a subshrub, which can grow up to reach 1 m in height, with its' stems often being lignified [1].



FIGURE 1: CATHARANTHUS ROSEUS PLANT

Catharanthus roseus leaves are simple and entire, and are disposed through the stems oppositely, merging from stems by a petiole, which can be green or red, that can have 3 to 11 mm long [1]. Leaves are normally blade elliptical or obovate, with lengths from 2 to 8 cm, and with widths from 1 to 4 cm [2]. Their adaxial surface is glossy green, and the

abaxial surface is pale green [2]. *C. roseus* flowers are hermaphroditic, and can acquire several tonalities, such as pink, white, purple, being even possible to develop 2 colours in the same flower. Flowers have 5 petals, which are slightly fused at the proximal end, surrounding the emergence of the 2 to 3 cm long cylindrical corolla tube, which widens near the top, at the stamen insertion [2].

1.1.2 Habitat, reproduction and life cycle

Vinca is well adapted to several different habitats, such as coastal and sandy ones, riverbanks, savannas, open forests, and scrubland [2]. Being easily propagated plants, *C. roseus* is often found in these locations' surroundings, such as roadsides, abandoned gardens or even farmlands, and it is sometimes considered to be harmful to some habitats, as *C. roseus* is related to be invasive [3].

Concerning its' reproductive biology, *C. roseus* is usually a monoicous plant, having complete flowers that allows self-pollination, although it is also common to happen crossed-pollination, which is dependent on the environmental conditions and the existence of pollination supporters, such as seasonal butterflies. However, there are some *C. roseus* cultivars that are not self-compatible [4]. Wild *C. roseus* populations are diploid, with $2n = 16$ chromosomes, number that do not vary with the cultivar, meaning that hybridization is viable between the different cultivars [5].

In normal environmental conditions, and after germination, *C. roseus* will take 6-8 weeks to initiate flowering and, when located in warmer climates, *C. roseus* can develop flowers and fruits all year [2]. Regarding *C. roseus* propagation, it usually occurs by seed, which fall in the nearby locations of the mother plant and are often transported by insects to other locations [4]. Seeds can remain in dormancy states for several weeks after achieving maturity, waiting for the optimal germination conditions, which includes temperatures between 20-25°C and an exposure to total darkness for about 20 hours [2].

1.1.3 Production methodology

Vinca is considered an easily developing plant, as it is not too demanding on its' environmental conditions, neither on its' needs regarding fertilization or water requirements [4]. As referred above, the optimal germination temperatures are between 20-25°C, which normally grants germination rates above 95% [2].

Normally, seedlings can be transplanted 3 weeks after germination and, if optimal conditions are provided, its cycle should be completed from 3 to 5 weeks after transplantation, when plants initiate flowering. Meanwhile, and depending on the purpose of the propagation, it can be necessary to prune the plant. *C. roseus* plants, being evergreen plants, can be maintained in production for approximately 2 years, the amount of time that corresponds to 2 life cycles. Concerning fertilization, *C. roseus* is not a demanding plant, being capable of developing in poor soil conditions [4].

Water management is an important factor in *C. roseus* production. Although this plant is considered to have some resistance to drought environments [4], it is important to maintain an equilibrium in water supply. In Vincas, water excess leads to leaves' senescence, by perturbing root's nutrient absorbing efficiency and consequently destabilizing the plant's metabolism (being chlorophyll production a crucial part of it). Also, an excessively humid atmosphere and substrate promotes fungal infections and the appearance of plagues. So, as *C. roseus* does not require great water supplies, it should be granted in low quantities.

Vinca production should be controlled in its' pests and diseases, as this plant is prone to be infested by *Tetranychus urticae* and *Phytophthora parasitica*, which causes root and stem rot [6]. Also, vinca is reported to be infected with "Malaysian periwinkle yellow", a disease that is caused by a phytoplasma, which is allocated in plants' phloem, where it lives and reproduces, damaging its host [7]. This disease is manifested by excessive yellowing of leaves, bunchy top and stunted flowers and leaves [8], and it can be transmitted to plants by several phloem-feeding Hemiptera families, which carry this phytoplasma in their salivary glands, and also by other plants [7].

1.1.4 *Catharanthus roseus* as a MAP

Although *C. roseus* is considered, and often produced as, an ornamental plant, its' real value relies on the properties that it possesses, as a medicinal plant. In fact, *C. roseus* is considered a Medicinal and Aromatic Plant – MAP, due to the medicinal and economic potential that it is contained on the secondary metabolites it produces. *C. roseus* produces around 130 different alkaloids, 40 of them being dimeric indole ones, which majority are composed by two monomeric molecules, vindoline (VDL) and catharanthine (CAT). Consequently, *C. roseus* produces, mainly concentrated in leaves [9], two dimeric terpenoid indole alkaloids - TIA (composed by an indole group and by an isoprene group) with great medicinal value and importance, namely Vinblastine (VBL) and Vincristine (VCR).

1.1.4.1 Vinblastine and vincristine as active components in oncologic treatments

These two dimeric alkaloids are used as the main active component in several commercialized chemotherapy drugs for numerous oncologic diseases [10].

VBL and VCR are TIAs with antineoplastic properties, characteristic that attributes themselves huge value. These secondary metabolites work as mitosis blockers, as they inhibit β -tubulin from being able to polymerize with α -tubulin, avoiding, therefore, microtubules formation [11]. With this action, VBL and VCR block the formation of the mitotic spindle, which leads to a dispersion of the chromosomes along the cytoplasm and, consequently, induce cell apoptosis [11].

With this induction of cell apoptosis, VBL and VCR inhibit one of the most important characteristics of tumoral cells, their neoplastic activity [12], which permits the undesirable rapid growth of tumours. VBL and VCR are considered pharmaceuticals since 1960 and 1963, respectively, and they are main components to treat several oncologic diseases [13].

Their importance is, therefore, huge towards oncologic treatments, and their value is compatible with their referred importance, as it is projected an increased incidence of oncologic diseases, not only in Portugal but in the world in general [14,15,16,17,18].

1.1.4.2 Other valuable *Catharanthus roseus* alkaloids

Catharanthus roseus is considered a valuable plant, partially because of VBL and VCR synthesis, but these two dimeric alkaloids are not the only valuable alkaloids produced in these plants. Ajmalicine and serpentine are two monomeric alkaloids that are mainly concentrated in *C. roseus* roots [19], which have antihypertensive, anti-depressive and anti-stress properties, being also highly valuable to medicinal purposes [20]. Their concentration in plants and consequent extraction efficiency are, therefore, also relevant to supply the needs that the pharmaceutical companies must provide to the community.

Hence, *C. roseus*'s medicinal and economic value relies on the unique properties of the TIA it produces, and on the potential that the plant has, by producing them.

1.2. Light-quality's influence on plants

Light has two crucial roles towards plants growth and development. In one hand, light is the source of energy used in photosynthesis, allowing plants to synthesize energetic biomolecules, which are applied in metabolic and physiological processes. On the other hand, light is a signal that induces several morpho-physiological mechanisms, regulating plants' growth and development [21]. However, not all light-spectrums are viable to these two roles, since light-spectrums with higher wavelengths (>800nm, infra-red wavelengths) do not possess the minimal required amount of energy to be absorbed by plants' photoreceptors, as it can be proven by the increased reflectance in near infra-red wavelengths when compared to red-light ones [22], making it useless to plants' physiological processes.

Photosynthetic active radiation – PAR – shows that different wavelengths are used by plants to photosynthesize, and to regulate their metabolism, thereby dictating their morphological and physiological processes, such as the regulation of the transpiration rate, stomatal motion, and also the synthesis of hormones and metabolites [23]. Within these several PAR wavelengths, red and blue light-spectrums are the most crucial ones, due to their importance to plants' life cycle, morphological and physiological mechanisms, metabolism and biomolecules synthesis [22]. However, some non-PAR light-quality also influences plants' behaviour and development. Ultraviolet (UV) light-spectrum is an aggressive one, due to the high amount of energy that the photons at these lower wavelengths possess, which tend to be harmful to cells and tissues, causing stress responses in plants [24].

1.2.1. The impact of light-quality on plants' morpho-physiology

As referred above, light-quality controls plants' morphological and physiological mechanisms. In fact, red, far-red and blue light-spectrums are directly linked with several morpho-physiological aspects in plants' life cycle, such as plant height, branching, seedling development, leaf expansion, seed germination, flowering and shade-avoidance responses, which traduces, therefore, in a huge influence towards plants' morphology and having a crucial role towards plants' physiological processes [23].

1.2.1.1. Red/far red light-spectrum's importance

Concerning red and far red light-spectrums, these two are absorbed by photoreceptors present in all plant tissues, also known as phytochromes. These phytochromes are divided into Phytochrome red (Pr – absorbs red light), and Phytochrome far red (Pfr – absorbs far red light). Red and far red light are antagonists, since red light absorption by

Pr causes it to acquire Pfr form, and far red light absorption by Pfr causes it to acquire Pr form [23].

This dynamic equilibrium, and the ratio between Pfr and Pr (which is normally calculated as Pfr/P_{total}) has a major importance in several morpho-physiological processes, such as flowering. In fact, values of this ratio located between 0.63 and 0.80 were proven to be more efficient in promoting flowering, when compared to ratios higher than 0.80 [23].

Referring to plants' vegetative growth, R-light induces the development of several lateral shoots, which are long, contrasting with the short main shoot [25]. Exclusive R-light treatments significantly increases shoot and root length, although an RB treatment had higher impact in fresh weight (FW) and dry weight (DW) in the same plant organs [26]. R-light treatments also cause a significantly lower content in chlorophyll a, and in total chlorophylls, when compared to RB treatments, and even when compared to B-light treatments. Furthermore, R-light/FR-light ratio controls several morpho-physiological characteristics, such as the already referred development of lateral shoots, but also germination, etiolation, petiole length and stem elongation (being promoted in low R-light/FR-light ratio and retarded in high ones [27]). These morphological parameters are directly influenced by the availability of Gibberellic acid (GA), which concentration is dependent on the plants' exposure to R-light and to this R-light/FR-light ratio. As already been proven, a high R-light/FR-light ratio (4,4 in the referred investigation) causes dwarfism, by inhibiting the biosynthesis of GA, and retards flowering [28].

1.2.1.2. Blue light-spectrum's importance

Concerning blue light-spectrum, it is absorbed by plants due to the presence of cryptochromes (CRY), and it has a crucial role in inducing flowering [22].

Referring to plants' vegetative growth, B-light represses plants' development, leading to the production of smaller, thicker and darker green leaves, but also reducing photosynthesis rate and total plant biomass, when comparing plants grown with and without a B-light treatment [29]. Furthermore, B-light is considered to be a growth regulator, due to its influence towards several morpho-physiological factors, such as phototropism, photomorphogenesis, leaf expansion, stomatal motion and chloroplast development [29]. Also, a study developed with *Carpesium triste*, clarified that a B-light treatment led to the development of plants with low vigour, low FW and DW, and shorter shoots and roots [26].

1.2.1.3. Ultraviolet light-spectrum's impact

As mentioned before, UV light-spectrum is harmful to plants. This specific wavelength is not usable by plants to collect the energy used in photosynthesis, and the energy of this

light quality is too high, causing damages to plants' cells and tissues [24]. However, UV wavelengths influence several biological processes in plants, such as morphology, physiology and metabolite accumulation [30]. Likewise, plants exposed to UV are reported to have some phenotypical alterations, being generally smaller, with smaller but thicker leaves, shorter petioles and shorter stem elongation [31]. Investigations developed with *Betula* species and with *Arabidopsis thaliana* highlighted the negative impact of UV-B light-spectrum towards leaf expansion and leaf size, repressing them [32;33]. However, these alterations in leaves' morphology to the exposure to UV-B appear to be temporary, as these conditions are reverted, due to the inducing of the plants' defence mechanisms that are triggered by the stress caused by UV-B. These defence mechanisms can even promote a compensatory growth, although being species-specific [31]. Nonetheless, UV light-spectrum's wavelength is crucial towards the impact that it may have on plants. Contrarily to UV-B, which causes a significant decrease in leaf, stem and root dry weights, leaf thickness and specific leaf area, UV-A is reported to increase leaf and root dry weights (even when compared with the control group, which was provided exclusively with PAR light), leaf thickness, stem dry weight and specific leaf area (when compared to UV-B light treatment) [34].

1.2.2. Alkaloid production and the role of light-spectrums

Light is considered one of the most important abiotic factors for plant development, being essential to regulate photomorphogenesis [35], controlling seed germination, hypocotyl elongation, and also in other plant development stages, such as regulating the development pattern and manipulating the production of several biomolecules [36]. Some of these biomolecules are secondary metabolites, which are not essential molecules to the growth and development of plants. However, they are important to their interaction with the environment. Alkaloids, being some of these secondary metabolites, are normally oriented to plants' defence mechanisms, mostly against herbivores, being highly toxic [37]. Due to their toxicity, they are often gathered in cells' vacuoles, in order to prevent negative effects to plants themselves [38]. However, alkaloids have other functions in plants, such as nitrogen storage, growth regulation, maintenance of the ionic balance and acting as detoxication agents [39].

C. roseus's CAT, VDL, VBL and VCR productions are, therefore, influenced by light and its different light-spectrums. For instance, near-ultraviolet light spectrum (N-UV) is an abiotic factor with high impact towards *C. roseus* VBL production, as it promotes a non-enzymatic reaction, which is one of the mechanisms already proven to increase VBL production (being the other an enzymatic reaction, which will not be studied in this work). N-UV significantly increases VBL availability in these plants, when provided both

naturally and artificially, specifically by promoting CAT oxidation. This important oxidation is catalysed by the increasing concentration of reactive oxygen species (ROS), which is promoted by the stress caused by N-UV in *C. roseus*. So, the impact that N-UV light has on CAT oxidation is appointed to be a trigger reaction to dimer alkaloid synthesis, non-enzymatically [40]. However, as Kazumasa *et al.* study also suggests, this availability is also dependent on the light spectrum applied in a pre-treatment, as a treatment based on the visible light spectrum, referred as BR (blue and red light spectrums) will increase the availability of CAT and VDL, and decrease the production of VBL, due to the absence of the triggering factor towards VBL production, the oxidation of CAT, catalysed by N-UV. Furthermore, an N-UV light-spectrum also influences the pathway in which CAT and VDL associate, to synthesize VBL, since it promotes the non-enzymatic association of the two MIA, procedure that is reported to be more effective in VBL synthesis and less costly [41]. Therefore, it is understood that VBL production is influenced by the exposure of plants to a specific quality of the light spectrum, N-UV, while the availability of CAT and VDL is enhanced by the exposure to blue light (B-light) and red light (R-light) spectrums.

Ultraviolet-B (UV-B) light spectrum is also referred to increase VBL and CAT productions in cell suspension cultures, alkaloids that are even suggested to be absorbing UV-B light and, therefore, avoiding the damages that the oxidative stress caused by it would provoke on plant cells [42]. Another study regarding UV-B light's impact on alkaloid production in *C. roseus* suspension cultures revealed that VDL and CAT production was also enhanced by a 5-minute treatment with UV-B [43].

Nonetheless regarding light spectrums, R-light treatments applied has showed to influence MIA alkaloids CAT and VDL synthesis, increasing their concentrations in both young and total leaves, when compared to white light (W-light) [44], and even when compared to B-light or an association of B-light and R-light [45]. R-light is reported to excite a phytochrome, which will induce DAT activity and, therefore, promote VDL synthesis [46], being also appointed that R-light spectrum alone is sufficient to induce VDL synthesis. However, it is also known that an excessive exposure of *C. roseus* to R-light, of approximately $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ causes a decrease in CAT and VDL concentrations, due to a decrease in chlorophyll content, which reduces plants' photosynthetic activity [47].

Light spectrums are, therefore, essential for the synthesis of the valuable alkaloids that *C. roseus* produces. Both red and N-UV light-spectrums have a crucial role in alkaloid production, complementing themselves through the metabolic pathway in which they are synthesized.

1.3. Plant factories

Plant factories are, more and more each day, one of the most viable options concerning plant production. The constant increase of the world's population, linked with the observed increasing extreme weather events, climate changes and even the increased concern with deforestation, biodiversity decrease, and excessive soil and water use, it is of major importance to apply production methodologies that increase productivity by maximizing production in small areas. Also, as it is observed a decrease in the "agricultural population", this "urbanization" of plant production could be a good answer to this decrease, which can be obtained by the development of these plant factories.

Plant factories answer positively to all the concerns already referred, as they can be built anywhere worldwide, independently of the region's light and soil conditions, and permits a more efficient use of the required resources, such as water, CO₂ and fertilizers, which diminishes pollutant emissions to the surrounding environment. This production methodology involves the application of artificial light (via fluorescent lamps and, more recently, light emitting diodes – LEDs), which can be manipulated and adapted to the plants that are aimed to be produced, and to the aims of the production. Furthermore, as all climatic conditions are monitored and manipulated, production can be developed all year, and even with productivities up to 100 times superior to the ones obtained in field productions [48]. The possibility to control abiotic factors permits not only the development of higher products (richer in nutrients, secondary metabolites, or other molecules depending on the objectives of the production), without the need to apply phytochemical products, but also the development of safer products, without the occurrence of diseases or plagues, and even with hugely lower incidence of bacterial infection (which permits a longer post-harvesting shelf life) [48].

Regarding anti-neoplastic alkaloids' production, the already referred increasing incidence of oncologic diseases, associated to the projected increased demand of oncological therapeutics (also due to the increased life expectancy), and concerning the higher and easier availability of products that plant factories develop, specially in urban areas (where is located the vast majority of the population), they should be considered one of the most viable options to develop a massive alkaloid production.

1.4. Problematics and challenges

Literature and scientific investigations already answered some of the questions and dynamics of the physiological mechanisms in *C. roseus* and their respective production of the valuable alkaloids CAT, VDL, VBL and VCR. However, the influence of some abiotic factors, mainly light-spectrum, in alkaloid production, are still major difficulties to overcome.

The importance that *C. roseus* possesses, reinforces the need to continuously study this plant, with the aim of improving the contribution that it can offer to the population, mainly by being a crucial part of the treatments to oncologic diseases that, as referred before, are expected to increase their incidence. Therefore, it is imperative to supply the pharmaceutical market with much higher quantities of these molecules than the amount that plants currently can provide. Consequently, with the low availability of the alkaloids used in chemotherapy in *C. roseus*, their extraction is too costly [18], promoting the production of synthetic molecules that will substitute the ones synthesized by the plant. Thus, the need to increase alkaloids natural production in *C. roseus* is essential to provide the population higher availability of the oncologic treatments that have in their composition VBL and VCR, and possibly lowering the economic costs of their production and, consequently, their final price [49].

As referred before, the interaction of the different light-spectrums with *C. roseus* has different results regarding CAT, VDL and VBL production by the plant. N-UV, UV-B and red light-spectrums clearly increase these alkaloids' availability in *C. roseus*, although promoting it by acting in different moments of the metabolic pathway in which they are synthesized.

There are some loopholes in the scientific knowledge concerning alkaloid production in *C. roseus*. For instance, there was not published and declared yet an ideal light treatment that maximizes alkaloid production in *C. roseus*, as the research already published have only tested light-spectrum treatments singularly, not associating different light-spectrums in the same treatment. Therefore, it is important to develop an experimental work that applies treatments that associate different light-spectrums, which will complement themselves throughout the TIA metabolic pathway, and that can prove to be highly efficient for VDL, CAT and VBL synthesis. Additionally, it is necessary to understand if alkaloid production varies among the different varieties of *C. roseus*, and to possibly declare a variety or varieties that are more productive and thus more prone to massively produce the therapeutic active components, in plant factory conditions.

1.5. Objectives

Concerning the problematics and challenges already referred, and considering the lack of knowledge that exists regarding *C. roseus* management towards alkaloid production, the objectives of this research englobe answering the following theoretical questions:

- How will an exposure to an N-UV light spectrum influence *C. roseus*' morpho-physiology?
- Which *C. roseus* cultivar(s) are more productive regarding their morpho-physiological parameters, both with and without an N-UV light spectrum, in plant factory conditions?

Answering these questions will permit to pinpoint the cultivar(s) that have a better morpho-physiological response when exposed to N-UV, that consequently are more prone to be analysed concerning their alkaloid production potential, which will be performed with N-UV light spectrums.

2. Materials and methods

2.1. Plant material and growth conditions

Catharanthus roseus plants, whose seeds were ordered from Sakata, and germinated in Valencia, were grown in three climate chambers, located at Viveiros Vitor Lourenço, in Maia, Porto. This experiment encompassed the setting of 3 different replicates, with 5 weeks duration each. Each replicate was developed in 3 chambers, in which one of them had a different light treatment from the other two.

2.1.1. Plants' selection and setting

In order to perform a screening, several *C. roseus* cultivars were included in the experiment, which were selected between some types, encompassing the following: Cora, which cultivars were Cora Deep Lavender (CDL), Cora Burgundy (BUR) and Cora Red (RED), Cora XDR, which cultivars were Cora XDR Punch (PUN), Cora XDR White (WHI) and XDR Polka Dot Apricot (PDA), Sunstorm, which cultivars were Sunstorm Apricot (APR) and Sunstorm Deep Lilac (SDL). Furthermore Titan type, with the cultivar Polka Dot (TIT), was also included. Also, a mix from the Pacifica type and Red Halo from the Sunstorm type were amongst the selected ones, but these two were not included in results (which reasons will be explained further), and so they will not be referred further on. The selection of these cultivars was based on the lack of knowledge that exists among the vast majority of them, and also on their morphological characteristics, such as the colour of their flowers. Here, cultivars were selected with the aim of being compared and, therefore, different cultivars with some morphological similarities between them were selected. Also, Sunstorm Apricot was selected due to being a cultivar whose genome was completely sequenced.

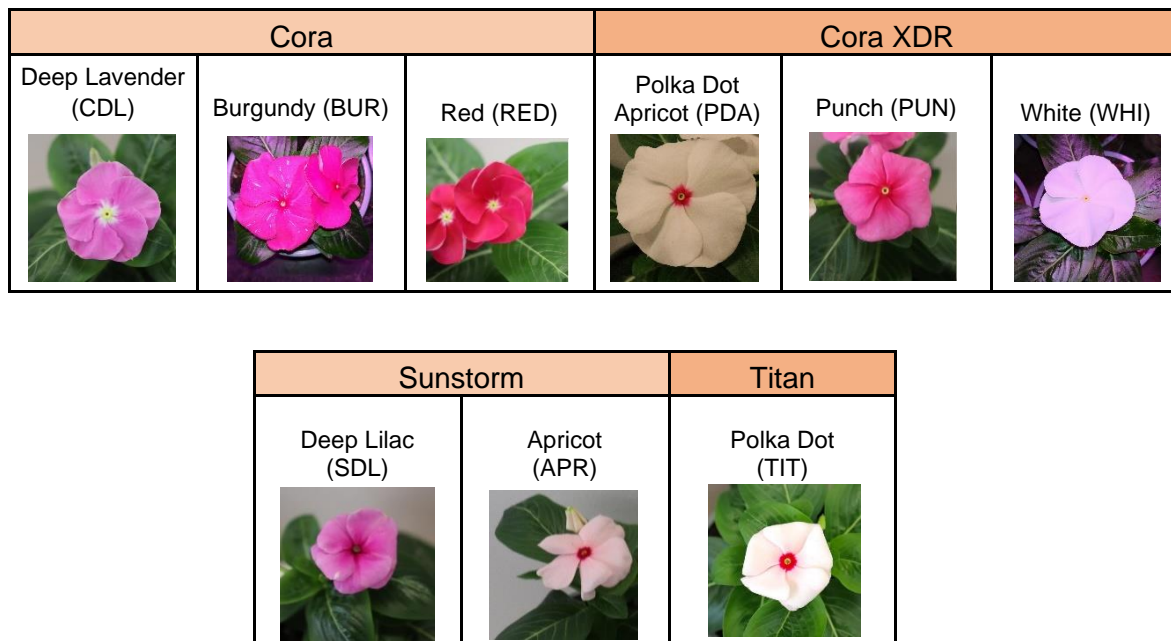


FIGURE 2: *C. ROSEUS* CULTIVARS IN THE EXPERIMENT, AND THEIR RESPECTIVE FLOWER'S PHENOTYPE

Plants were transplanted to 8cm in diameter vases and were consequently set in boards located at the chambers (as showed in figure 3), at the physiological stage of plantlets, moment in which each of the trials begun. Their distribution along the board was randomized, by setting the plants in cycles, in a defined order in each chamber (one plant per cultivar, per cycle). With this setting, it was possible to grant that there was no proximity between 2 plants of the same cultivar.

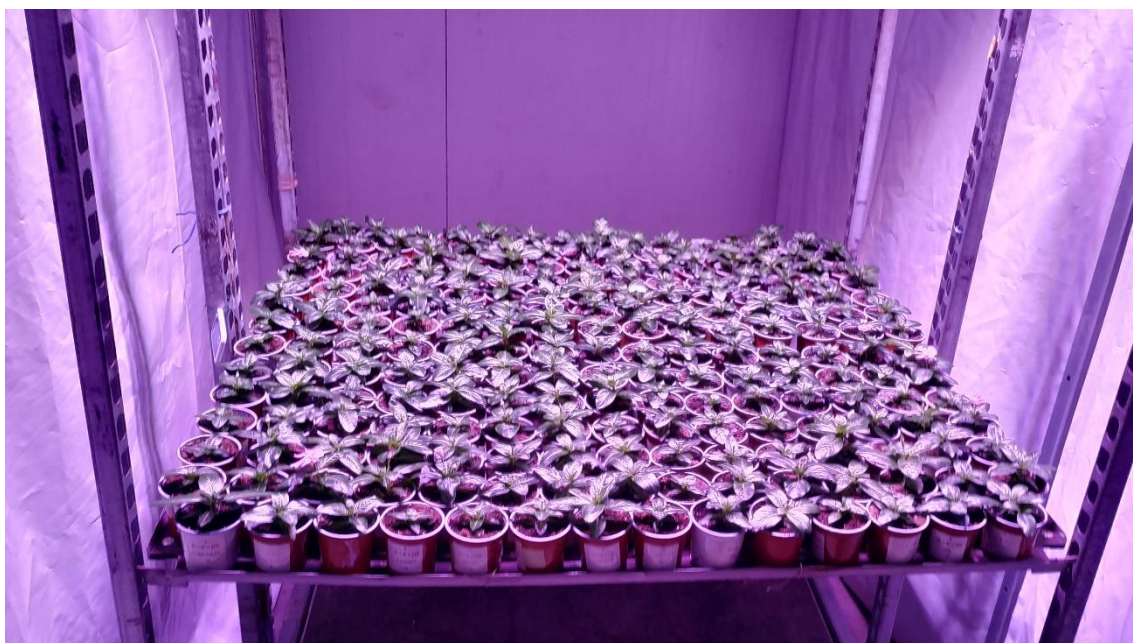


FIGURE 3: EXAMPLE OF ONE OF THE CHAMBERS SET FOR THE EXPERIMENT. TWO BOARDS WERE FILLED WITH THE NEEDED NUMBER OF PLANTS, TO PERFORM THE EXPERIMENT;

2.1.2. Light treatments' setting

The light treatments applied to plants during this investigation were always composed by RB light spectrums, in a 12h photoperiod (beginning at 7:00 and ending at 19:00), which was defined as control. This light treatment had a proportion of 3:1 (R:B). In each replicate, in one or two chambers, it was provided an extended 2h light period of an exclusive N-UV light (beginning at 19:00 and ending at 21:00).

Light's intensity along the boards was monitored weekly, measured by an illuminance spectrophotometer, granting, to all plants in the experiment, a light intensity in accordance with the following:

- **R+B (A)** – Border area: 95 – 110 $\mu\text{mol}/(\text{m}^2\text{s})$; Central area: 140 – 170 $\mu\text{mol}/(\text{m}^2\text{s})$
- **N-UV (B)** – Border area: $272 \pm 17 \text{ mW}/\text{m}^2$; Central area: $328 \pm 31 \text{ mW}/\text{m}^2$

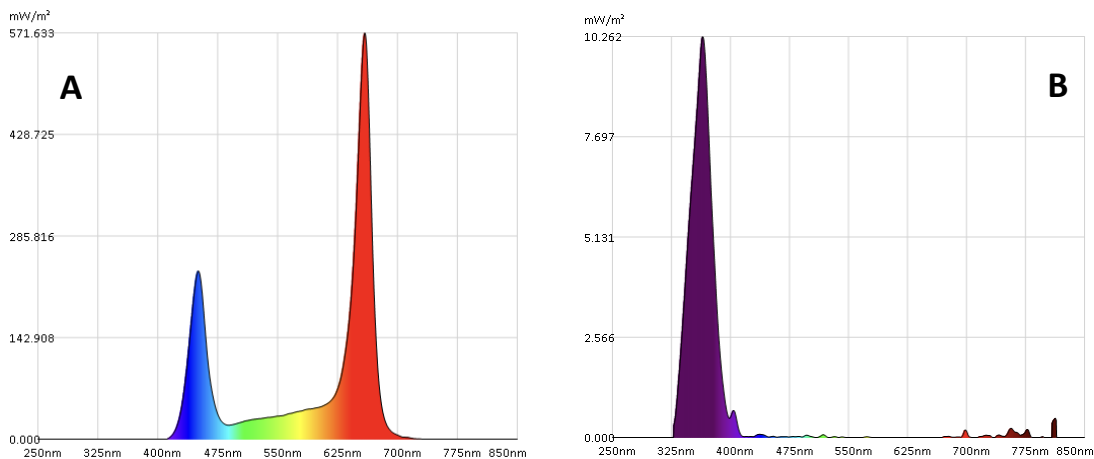


FIGURE 4: LIGHT SPECTRUM'S WAVELENGTH AND RESPECTIVE INTENSITY OF THE DIFFERENT LIGHT QUALITIES PROVIDED. A- R+B LIGHT-SPECTRUMS; B- N-UV LIGHT-SPECTRUM;

2.1.3. Temperature and humidity monitoring

The climate chambers were set with a ventilator and programmed for the ideal temperatures for *C. roseus*. With this setting, temperatures always oscillated between 23°C and 25°C during the entire experiment, conditions that were also monitored, in this case continuously. Also, relative humidity (approximately 80%) was monitored in a constant basis.

2.2. Experimental design

The setting of this experiment included the development of 3 different replicates, which differ in the number of plants and cultivars in each chamber, and in the presence or absence of the UV lamps in each of the chambers. With this setting, it was possible to grant 3 replicates of each cultivar (except for APR, which was exposed to an RB + N-UV light-treatment in 2 replicas), in both light-treatments. At the end of the first and second replicas, UV lamps were transferred to another chamber, with the objective of mitigating the possible effects of the chamber's location on results.

2.2.1. *C. roseus* plants development, first replicate

The first replicate was initialized on June 21st, and englobed 52 plants of each of the cultivars, with a total of 208 plants (per chamber). Here it were set in chamber A, PAC, CDL, TIT and SRH cultivars, in chamber B were set WHI, BUR, PDA and SDL cultivars, and in chamber C were set PAC, CDL, TIT and SRH cultivars. A and B chambers had the control light treatment, and chamber C had RB + N-UV light treatment. The first trial closure corresponded to the end of the 5th week of the experiment (on the 25th of July).

2.2.2. *C. roseus* plants development, second replicate

The second replicate was initialized on the 26th of July, with some alterations in the setting of the chambers. Here, all chambers were set with CDL, TIT, BUR, WHI, PDA, SDL, RED and PUN cultivars, with 26 plants of each one. PAC and SRH were forfeited, due to the fact that PAC was a mix, being, therefore, highly unpredictable, and SRH, due to the huge susceptibility to be infected by *Phytophthora parasitica*. In chambers A and C, plants were exposed to the control treatment, and B plants were exposed to RB + N-UV.

In this trial, with the diminished number of plants by cultivar, which was reduced to half to permit a complete analysis of every cultivar in the experiment, and with the need to use almost all plants of each of the cultivars, the sample was stratified in blocks. In each of the chambers, the 2 supportive boards were subdivided into 6 different areas each, by a wire. These 6 different areas were rotated every 3 days to the consecutive position. With this stratification and rotation, the objective was to nullify the availability of bedding plants, which enables the use of the totality of the plants in development. This second trial completion corresponded to the end of the 5th week, on the 28th of August.

2.2.3. *C. roseus* plants development, third replicate

The third replicate was initialized on the 2nd of September, and had 2 chambers (A and C) with the RB + N-UV treatment. Chamber B was set exclusively with the RB light-spectrum treatment. In this replicate, chamber A was set with CDL, BUR, SDL, RED,

WHI, PDA, PUN and APR. In chamber B, with the need to create 3 replicates of the cultivar introduced in this replicate, APR, it was divided into 3 groups of plants, which were treated as different cultivars, being set carefully, avoiding any association between them. APR had, therefore, 3 different groups (referred from now on as B1, B2 and B3) of 26 plants, each. Adding to B1, B2 and B3, in chamber B, TIT, CDL, SDL, RED and PUN were also set. In chamber C, TIT, BUR, SDL, RED, WHI, PDA, PUN and APR were the selected varieties to be set.

This third replicate was completed on the end of the 5th week, on the 6th of October.

2.2.4. Sample collection

At the end of all three trials, and concerning the future works related to the global project, it was performed a sample harvesting. This harvesting englobed 2 different procedures, which will correspond to 2 different quantifications. Firstly, 5 plants of each cultivar in each chamber were selected, and their flowers and leaves were collected, and immediately stored at -20°C. This harvesting will be applied in the quantification of the total alkaloid content of these plants. Secondly, with the objective of quantifying alkaloid concentration, gene expression associated to the alkaloid production, and protein quantification, 15 plants of each cultivar in each chamber were also selected, and leaves (fully extended and fully exposed ones) and flowers were collected, forming 5 experimental units, each one with a span of 3 different plants. Upon harvesting, they were immediately stored in liquid nitrogen. Consequently, these samples were stored at -80°C for further analysis.

2.3. Morphological and physiological analysis

2.3.1. Chamber plants monitorization

As referred before, at the beginning of each replicate, 5 plants of each of the cultivars set in each of the chambers were selected, with the objective of monitoring their growth and development, concerning attributes such as height, number of leaves (counting as a leaf the ones that had a minimum area $\approx 2\text{cm}^2$), number of nodes and also the reproductive development stage, number of flowers and number of floral buds. The monitoring of these parameters was performed weekly, inside the chambers, assuring that these plants were never deprived from the light-treatments set for them. With this monitoring, it was possible to understand the rate of growth of the cultivars and the different growth rhythm between them. These selected plants were monitored throughout the 5 weeks of each trial and, at the end of these 5 weeks, other parameters such as fresh and dry weights (of leaves, roots, flowers and stems) leaf area (measured by a Leaf Area Meter, LI 3100C, LI-COR), photosynthetic rate (determines the quantity of CO_2 consumed by m^2 of leaf, per second - measured with IRGA, LI-6400XT, LI-COR) and gas exchange rate (determines the difference of the CO_2 flowing into the leaf chamber and the CO_2 flowing out of the chamber - measured with IRGA, LI-6400XT, LI-COR), and relative chlorophyll content (measured with SPAD 502 plus Chlorophyll Meter, calculated by an average of 3x3 readings – 3 measures in 3 leaves), were added to the weekly ones.

2.3.2. Flowering monitorization

During the replicates, flowering was monitored. This monitoring occurred twice per week and included a registration of the physiological stage of all plants in each chamber. This registration was divided into four stages, being them “floral bud”, “intermediate”, “advanced” and “flowering”, which were identified as showed in figure 4.

Also, once a plant reached the “flowering” stage, the new floral buds developed by it were quantified. With this monitorization, it was possible to understand which cultivars entered the “flowering” stage faster, which were more productive concerning their flower production, and it was also possible to understand if the different light-treatments had an impact on flowering.

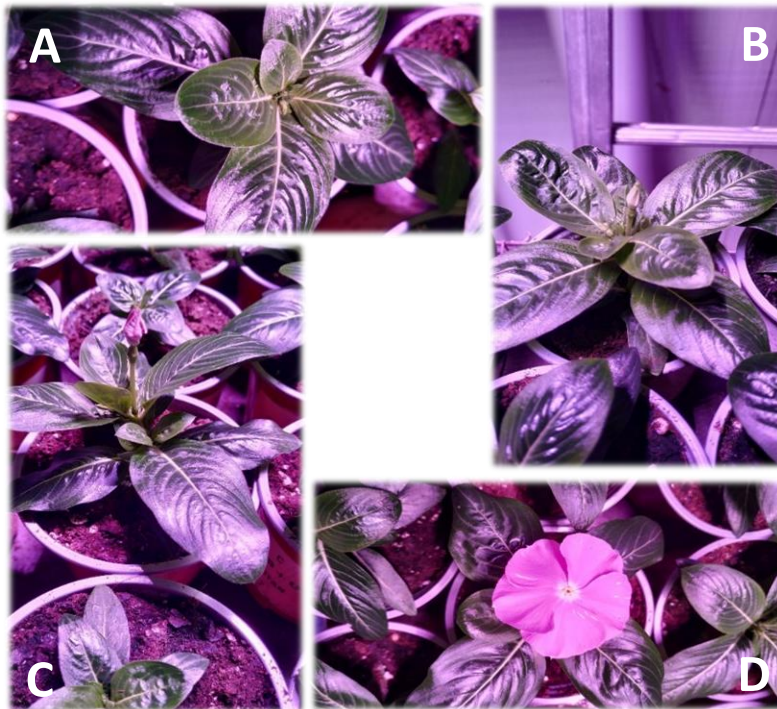


FIGURE 5: PHENOLOGICAL STAGES REGISTERED IN FLOWERING MONITORING; A- FLORAL BUD; B- INTERMEDIATE; C- ADVANCED; D- FLOWERING;

2.4. Statistical analysis

In this experiment, statistical analysis was performed with IBM's SPSS Statistics. All data in analysis was previously tested for the possibility of outlier occurrence, which were eliminated from the data once were identified. Also, all data-groups analysed were tested for its normal distribution, which graphics can be observed in the annex chapter. Also, Levene's tests were performed to assure the homogeneity of the collected data.

Two-way ANOVAs were performed, in normal distributions, to compare the two factors in study, "cultivar" and "light quality", and the crossing between the two. Consequently, when significantly different results were detected with ANOVA ($p \leq 0,05$), post-hoc Tukey HSD tests were performed. To properly analyse parameters with non-normal distributions, non-parametric Mann-Whitney ($p \leq 0,05$) and Kruskal-Wallis ($p \leq 0,05$) tests were performed.

3. Results

3.1. Plant morphology

3.1.1. Plant height

Concerning plant height, it was not observed a significant difference in the crossing between the factors “cultivar” and “light quality”. However, it was possible to observe a significant difference for the “cultivar” factor, as showed in the following graphic:

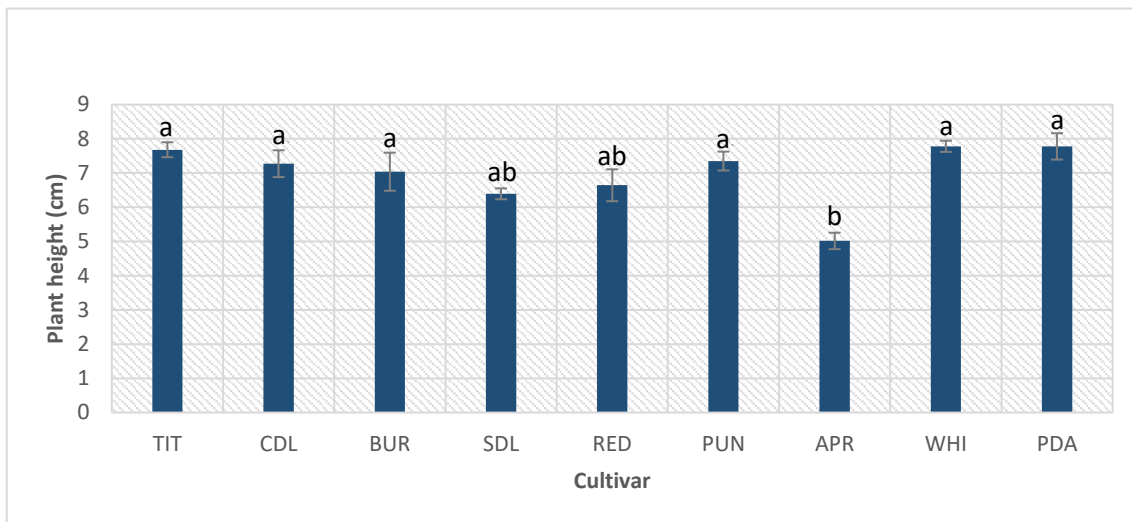


FIGURE 6: AVERAGE HEIGHT (CM) AND ASSOCIATED STANDARD ERROR, OF THE DIFFERENT CULTIVARS AT THE END OF THE 5TH WEEK. RESULTS REFERABLE TO ALL SELECTED PLANTS (CONTROL + N-UV). DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS (P=0,000)

With these results, it is possible to observe that the factor cultivar had an impact towards plant height, in which the cultivars TIT ($7,68 \pm 0,219$ cm), WHI ($7,78 \pm 0,163$ cm) and PDA ($7,78 \pm 0,385$ cm) had a significantly higher growth, when compared to APR ($5,02 \pm 0,241$ cm). APR has also showed to be statistically smaller than CDL ($7,27 \pm 0,392$ cm), PUN ($7,35 \pm 0,276$ cm) and BUR ($7,04 \pm 0,557$ cm).

3.1.2. Number of leaves and leaf area

3.1.2.1. Number of leaves

Concerning the number of leaves per plant, the obtained results did not show a significant difference for the crossing between “cultivar” and “light quality”. However, it is observable a significant difference for the “light quality” factor and for the “cultivar” factor. Concerning “light quality” factor, the results are showed in the following graphic:

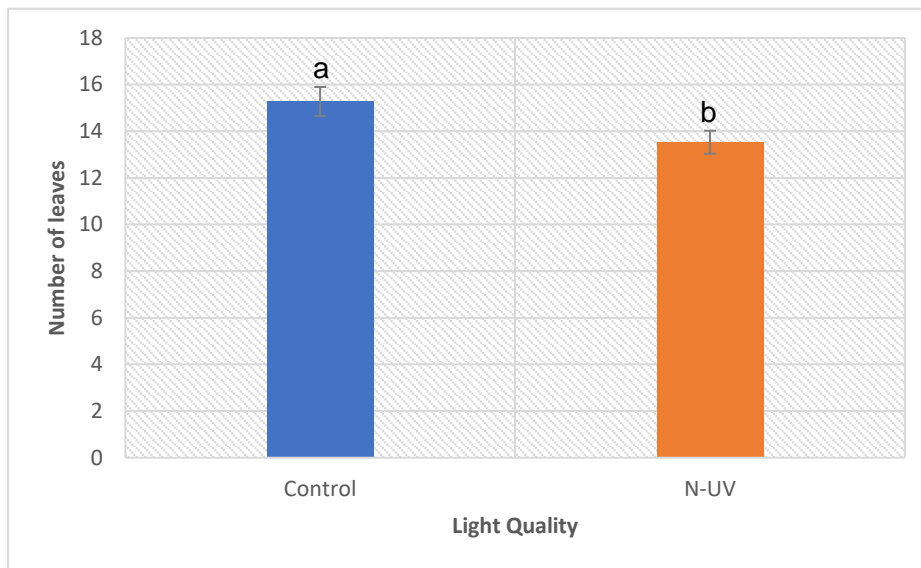


FIGURE 7: AVERAGE NUMBER OF LEAVES AND ASSOCIATED STANDARD ERROR, OF THE DIFFERENT LIGHT QUALITIES, AT THE END OF THE 5TH WEEK. DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS (P=0,010)

With these results, it is possible to observe that the “light quality” factor had an impact towards number of leaves, in which the control light quality (15,28 ± 0,620) traduced in a significantly higher number of leaves, when compared to the N-UV light quality (13,53 ± 0,493).

Concerning the calculated significantly different results in the “cultivar” factor, the following results were obtained:

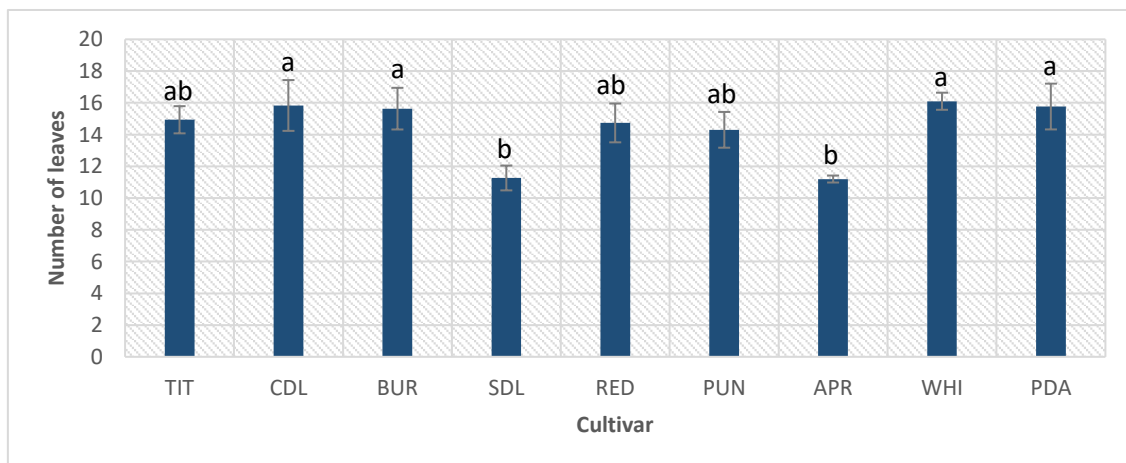


FIGURE 2: AVERAGE NUMBER OF LEAVES AND ASSOCIATED STANDARD ERROR, OF THE DIFFERENT CULTIVARS AT THE END OF THE 5TH WEEK. RESULTS REFERABLE TO ALL SELECTED PLANTS (CONTROL + N-UV). DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS (P=0,001)

These results reveal a significantly higher number of leaves in PDA (15,77 ± 1,442), WHI (16,10 ± 0,539), CDL (15,83 ± 1,600) and BUR (15,63 ± 1,312), when compared to SDL

(11,27 ± 0,718) and APR (11,20 ± 0,219). TIT (14,93 ± 0,856), RED (14,73 ± 1,223) and PUN (14,30 ± 1,126) did not have significantly different results.

3.1.2.2. Leaf area

Concerning leaf area – LA, statistical analysis did not show a significative difference for the “cultivar” and “light quality” crossing. However, it is observable a significative difference for the “cultivar” factor. Concerning “cultivar” factor, the results are showed in the following graphic:

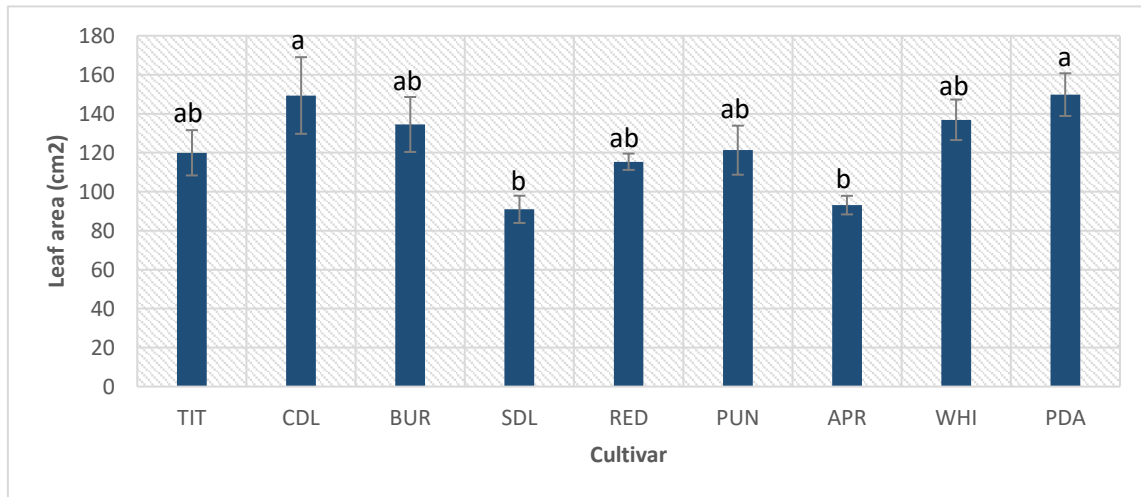


FIGURE 3: AVERAGE LEAF AREA (CM²) AND ASSOCIATED STANDARD ERROR, OF THE DIFFERENT CULTIVARS AT THE END OF THE 5TH WEEK. RESULTS REFERABLE TO ALL SELECTED PLANTS (CONTROL + N-UV). DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS (P=0,001)

With these results, it is possible to observe that the factor “cultivar” had an impact towards plant total leaf area, in which the cultivars PDA (149,80 ± 10,95 cm²), and CDL (149,35 ± 19,66 cm²) had a significantly higher growth, when compared to SDL (90,94 ± 6,99 cm²) and APR (93,11 ± 4,77 cm²). WHI (136,88 ± 10,40 cm²), BUR (134,49 ± 14,10 cm²), TIT (119,95 ± 11,62 cm²), RED (115,36 ± 4,19 cm²) and PUN (121,29 ± 12,58 cm²) did not have statistically significative results.

3.1.2.3. Specific leaf area

Concerning specific leaf area – SLA, the obtained results showed that there is no statistically different SLA, neither for “cultivar” and “light quality” crossing, nor for “cultivar” factor. However, it is observable a statistically significant difference between the “light quality” factor, as showed in the following graphic:

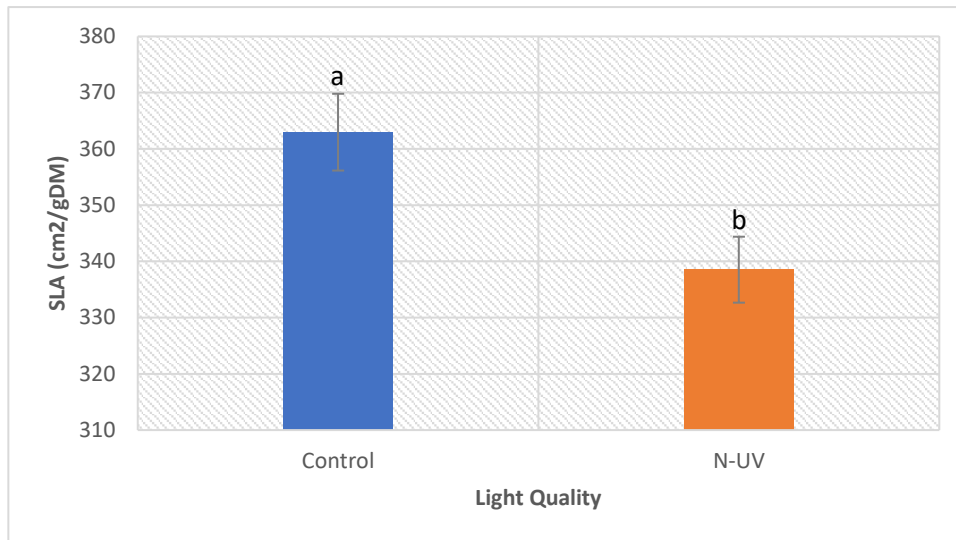


FIGURE 10: AVERAGE SLA IN BOTH CONTROL AND N-UV LIGHT QUALITIES AND ASSOCIATED STANDARD ERROR, AT THE END OF THE 5TH WEEK. DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED LIGHT QUALITIES (P=0,006)

With these results, it is possible to observe that the factor “light quality” had an impact towards plant SLA, in which the control group (362,97 ± 6,83 cm²/gDM) had statistically higher SLA when compared to the N-UV group (338,52 ± 5,86 cm²/gDM).

3.1.3. Plant biomass

3.1.3.1. Total dry matter

Concerning total dry matter, statistical analysis did not show a significative difference for the “cultivar x light quality” crossing. However, it was observed a statistically difference in plants concerning “cultivar” factor, as showed in figure 11:

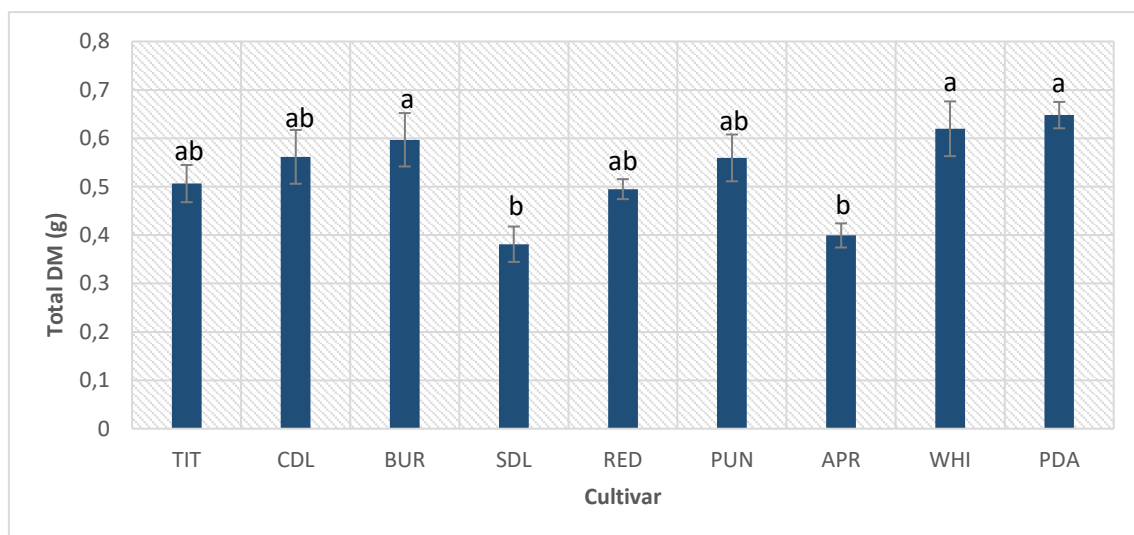


FIGURE 11: AVERAGE DMs AND ASSOCIATED STANDARD ERROR, OF THE DIFFERENT CULTIVARS (G) AT THE END OF THE 5TH WEEK. RESULTS REFERABLE TO ALL SELECTED PLANTS (CONTROL + N-UV). DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS (P=0,000)

With these results, it is possible to observe that the factor “cultivar” had an impact towards plant dry weight, in which the cultivars WHI ($0,620 \pm 0,0566$ g), BUR ($0,597 \pm 0,0552$ g) and PDA ($0,648 \pm 0,0298$ g) had a significantly higher weight, when compared to SDL ($0,381 \pm 0,0365$ g) and APR ($0,399 \pm 0,0279$ g). TIT ($0,506 \pm 0,0384$ g), CDL ($0,562 \pm 0,0556$ g), RED ($0,495 \pm 0,0206$ g) and PUN ($0,559 \pm 0,0483$ g) did not have statistically significant results.

3.1.3.2. Leaves dry matter

Concerning leaves dry matter, statistical analysis showed a significant difference for the “cultivar x light quality” crossing, as observed in figure 12:

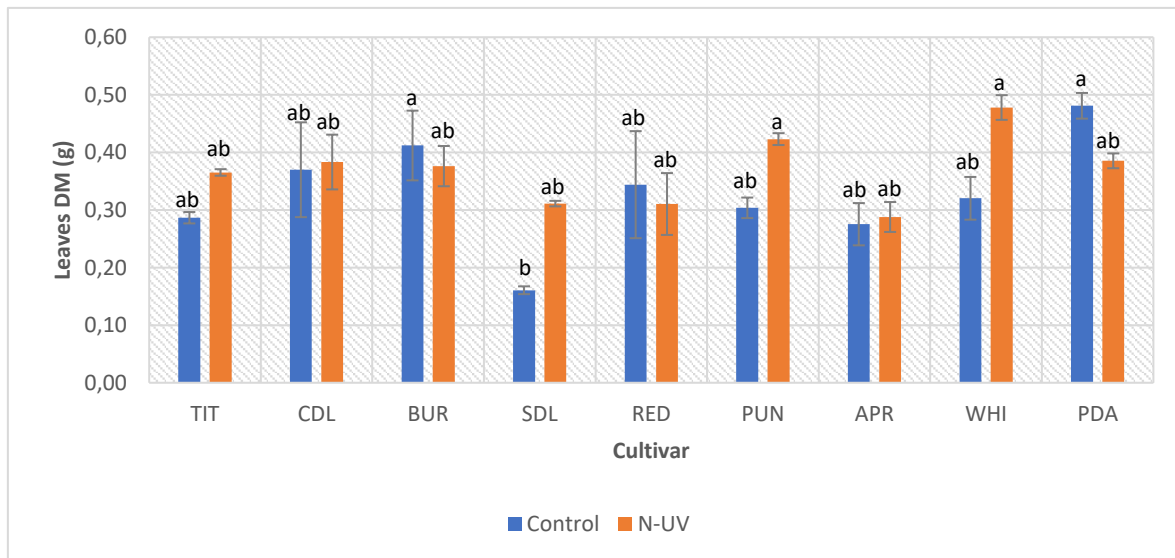


FIGURE 4: AVERAGE LEAVES DM AND ASSOCIATED STANDARD ERROR, OF THE DIFFERENT CULTIVARS (G) IN EACH LIGHT QUALITY, AT THE END OF THE 5TH WEEK. DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS (P=0,045)

With these results, it is possible to observe that PDA ($0,481 \pm 0,0223$ g) and BUR ($0,412 \pm 0,0605$ g) from the control group, and WHI ($0,478 \pm 0,0371$ g) and PUN ($0,423 \pm 0,0103$ g) from the N-UV group, had a significantly higher dry matter in their leaves, when compared to SDL ($0,161 \pm 0,00480$ g) from the control group. All the other cultivars with the respective treatments did not have significant results.

3.1.3.3. Flowers dry-matter

Concerning leaves dry matter, statistical analysis revealed a significant difference for the crossing between “cultivar” and “light quality”, as showed in figure 13:

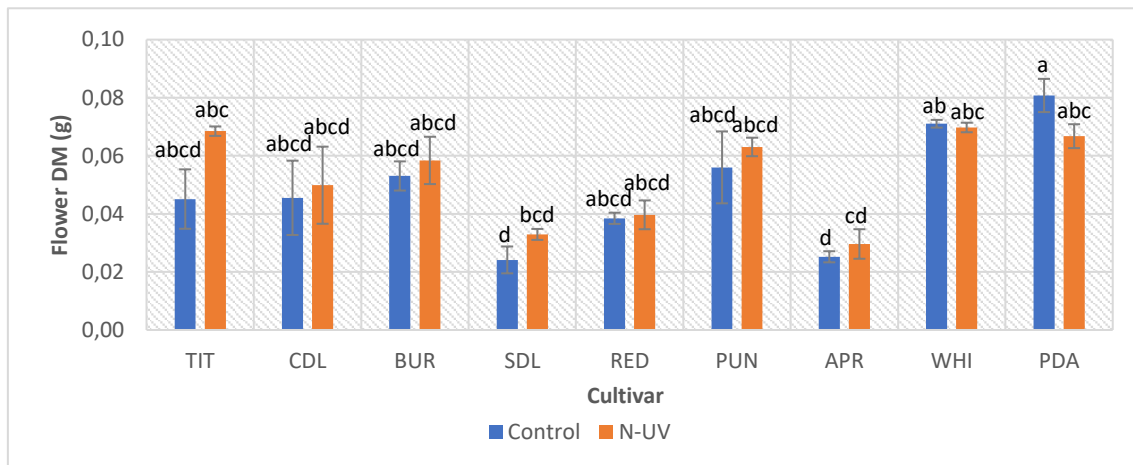


FIGURE 5: AVERAGE FLOWER DM (G) AND ASSOCIATED STANDARD ERROR, OF THE DIFFERENT CULTIVARS IN EACH LIGHT QUALITY, AT THE END OF THE 5TH WEEK. DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS (P=0,036)

With these results, it is possible to observe that the “cultivar x light quality” crossing had an impact towards flowers dry matter, in which the PDA from the control group ($0,0808 \pm 0,00990$ g) had significantly higher flower DM, when compared to both SDL ($0,0241 \pm 0,00801$ g – control; $0,0329 \pm 0,00328$ g – N-UV) and APR ($0,0252 \pm 0,00331$ g – control; $0,0296 \pm 0,00719$ g – N-UV) groups. These two cultivars, APR and SDL, were also statistically inferior in flower DM, when compared to WHI from the control group ($0,0710 \pm 0,00231$ g). However, most of the cultivars in both light qualities did not have statistically significant results.

3.1.3.4. Root/shoot ratio

Concerning root/shoot ratio, statistical analysis showed that there is no significant differences to be evidenced, for neither of the factors or their crossing, as showed in the following graphic:

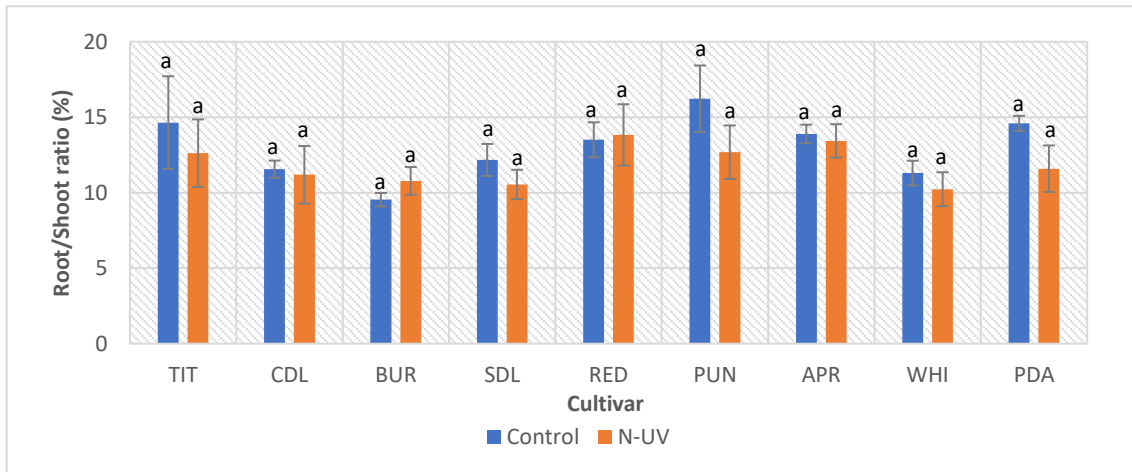


FIGURE 6: AVERAGE ROOT/SHOOT RATIOS AND ASSOCIATED STANDARD ERROR, OF THE DIFFERENT CULTIVARS (%) IN EACH LIGHT QUALITY, AT THE END OF THE 5TH WEEK. DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS

3.1.4. Number of produced flowers

Concerning the number of produced flowers, the obtained results showed a significative difference for the “cultivar x light quality” crossing, as showed in figure 15:

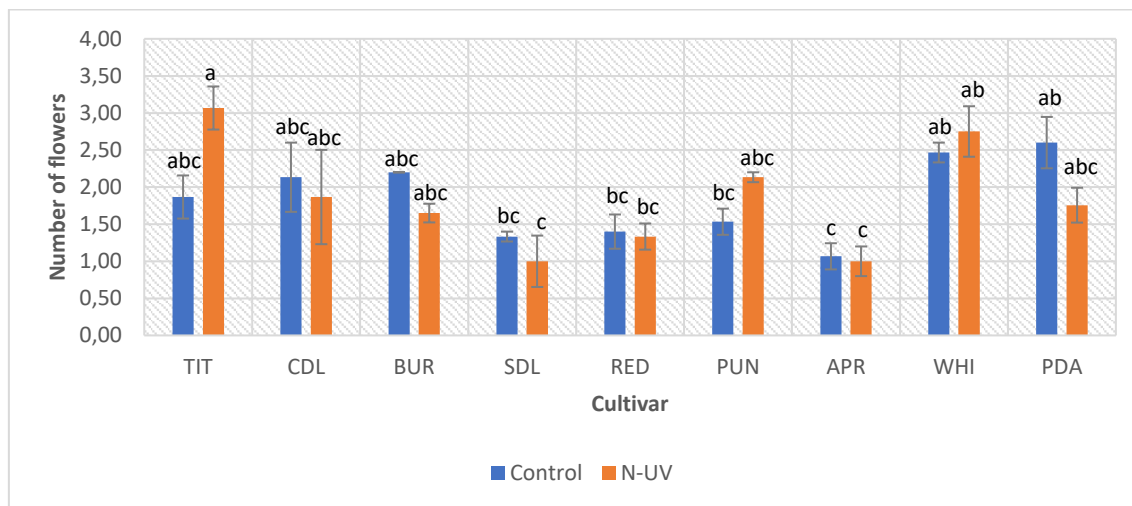


FIGURE 15: AVERAGE NUMBER OF PRODUCED FLOWERS AND ASSOCIATED STANDARD ERROR OF THE DIFFERENT CULTIVARS IN EACH LIGHT QUALITY, AT THE END OF THE 5TH WEEK. DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS (P=0,036)

With these results, it is possible to observe that the “cultivar x light quality” crossing had an impact towards flower production, in which the cultivar TIT from the N-UV light quality ($3,07 \pm 0,291$ un.) was significantly higher when compared to APR, SDL and RED in both light qualities in analysis, but also when compared to PUN from the control light quality ($1,53 \pm 0,176$ un.), although the control group for TIT ($1,87 \pm 0,291$ un.) was not. It is also important to pinpoint that APR in both light qualities ($1,07 \pm 0,176$ un. – R+B; $1,00 \pm 0,200$ un. – R+B+N-UV) and SDL from the N-UV light quality ($1,00 \pm 0,346$ un.) were significantly less productive in its’ flower number when compared to WHI from the N-UV light quality ($2,75 \pm 0,340$ un.) and to PDA from the control light quality ($2,60 \pm 0,235$ un.).

3.1.5. Flowering time

Concerning flowering time, data analysis revealed that there were no statistically different results for the “cultivar x light quality” crossing. However, it is observable a significative difference concerning the “cultivar” factor, as showed in figure 16:

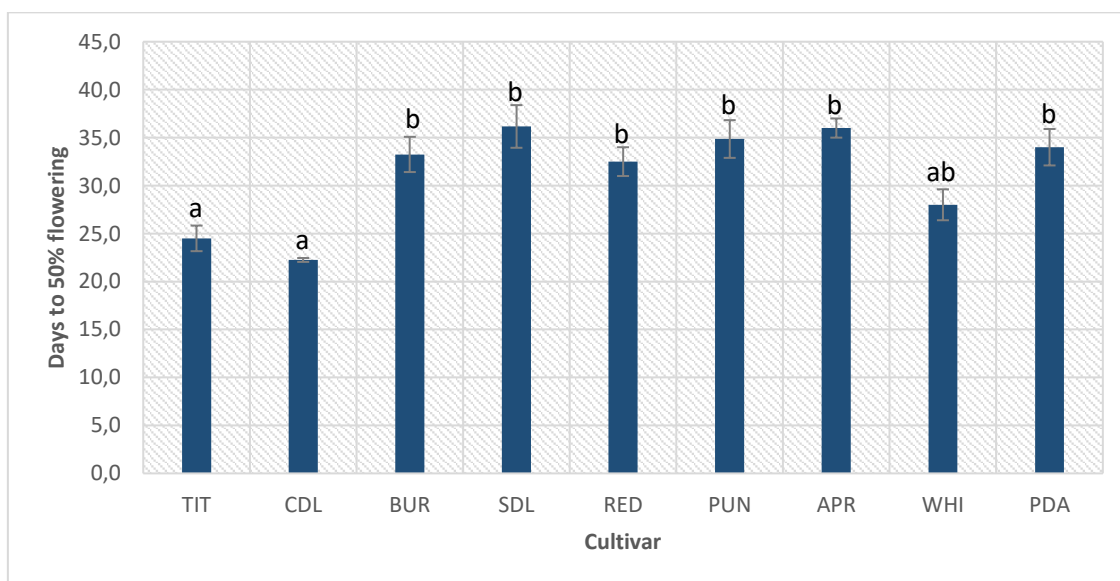


FIGURE 16: AVERAGE FLOWERING TIME AND ASSOCIATED STANDARD ERROR, OF THE DIFFERENT CULTIVARS AT THE END OF THE 5TH WEEK. RESULTS REFERABLE TO ALL SELECTED PLANTS (CONTROL + N-UV). DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS (P=0,000)

With these results, it is possible to observe that the factor “cultivar” had an impact towards flowering time, in which the cultivars TIT ($24,5 \pm 1,335$ days) and CDL ($22,3 \pm 0,204$ days) had a significantly faster flowering, when compared APR ($36,0 \pm 1,00$ days) and SDL ($36,2 \pm 2,22$ days), RED ($32,5 \pm 1,50$ days), PDA ($34,0 \pm 1,90$ days), BUR ($31,8 \pm 2,07$ days) and PUN ($34,9 \pm 1,86$). WHI ($28,0 \pm 1,61$ days) did not have statistically significative results.

3.2. Plant physiology

3.2.1. SPAD Index

Concerning SPAD index, and after performing a statistical analysis, results show that there are no significant differences to be observed for the “cultivar x light quality” crossing, neither for “cultivar” or “light quality” factors, as is showed in the following graphic:

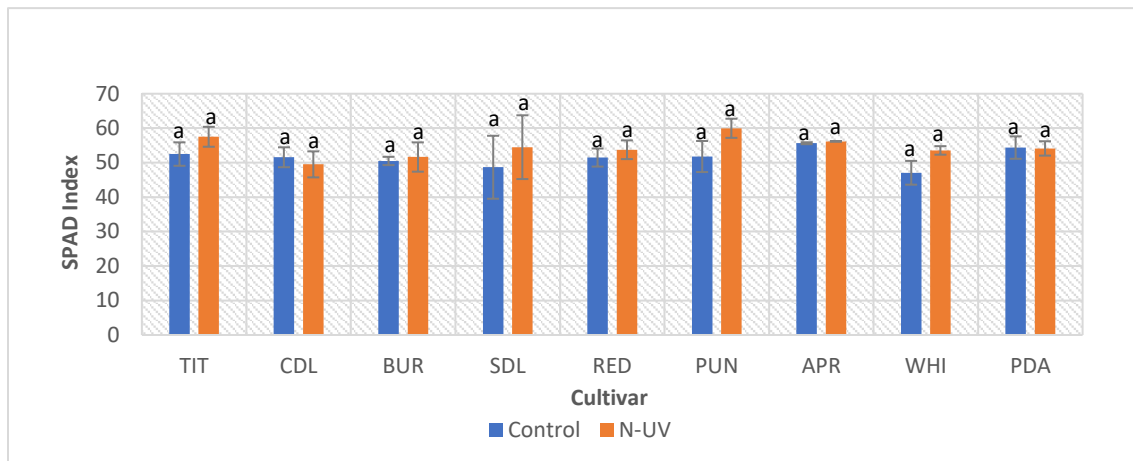


FIGURE 17: AVERAGE SPAD INDEX, FOR EACH CULTIVAR AT THE END OF THE 5TH WEEK OF GROWTH, IN EACH LIGHT QUALITY

3.2.2 Photosynthetic rate

Concerning photosynthetic rate (A), statistical analysis showed that there is no significant differences to be pinpointed for the “cultivar x light quality” crossing. However, it is observable a statistically significant difference concerning the “cultivar” factor, as showed in figure 18:

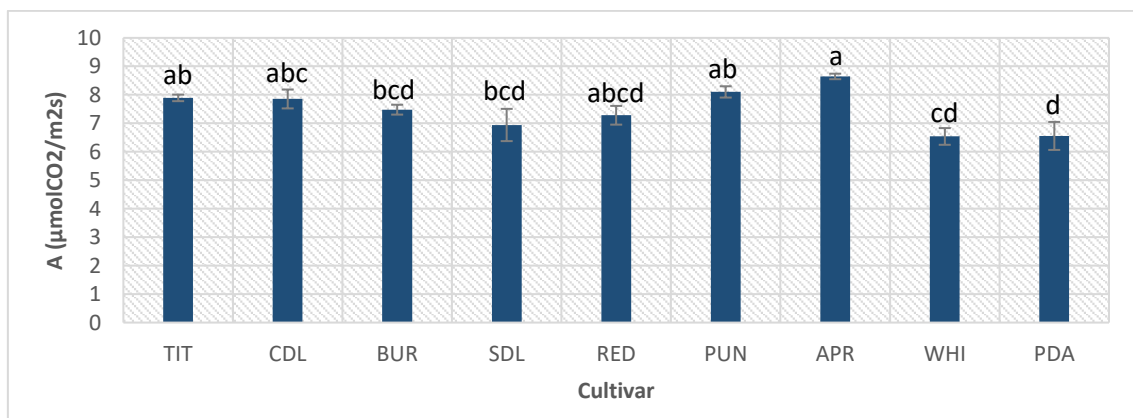


FIGURE 18: AVERAGE PHOTOSYNTHETIC RATE(μMOLCO₂/M²S) AND ASSOCIATED STANDARD ERROR, OF THE DIFFERENT CULTIVARS AT THE END OF THE 5TH WEEK. RESULTS REFERABLE TO ALL SELECTED PLANTS (CONTROL + N-UV). DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS (P=0,000)

With these results, it is possible to observe that the factor “cultivar” had an impact towards photosynthetic rate, in which the cultivar APR ($8,65 \pm 0,0968$ un.) had a significantly higher photosynthetic rate, when compared to PDA ($6,56 \pm 0,492$ un.), WHI ($6,54 \pm 0,294$ un.), SDL ($6,94 \pm 0,567$ un.) and BUR ($7,48 \pm 0,173$ un.). PDA was also less productive than TIT ($7,89 \pm 0,115$ un.), CDL ($7,85 \pm 0,331$ un.) and PUN ($8,10 \pm 0,198$ un.). RED ($7,28 \pm 0,329$ un.) did not have statistically significant results.

3.2.3. Gas exchange rate

Concerning CO₂ exchange rate (Ci), statistical analysis showed that there is no significant differences to be pinpointed for the “cultivar x light quality” crossing. However, it is observable a statistically significant difference concerning the “cultivar” factor, as showed in figure 19:

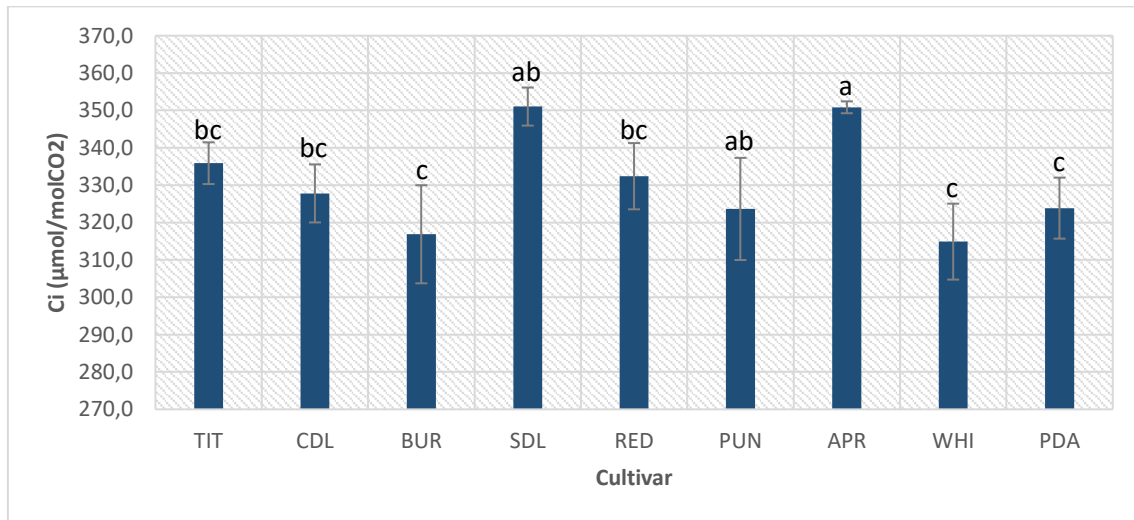


FIGURE 19: AVERAGE GAS EXCHANGE RATE ($\mu\text{MOL/MOLCO}_2$) AND ASSOCIATED STANDARD ERROR, OF THE DIFFERENT CULTIVARS AT THE END OF THE 5TH WEEK. RESULTS REFERABLE TO ALL SELECTED PLANTS (CONTROL + N-UV). DIFFERENT LETTERS CORRESPOND TO SIGNIFICANTLY DIFFERENT RESULTS BETWEEN THE REFERRED CULTIVARS ($P=0,018$)

With these results, it is possible to observe that the factor “cultivar” had an impact towards gas exchange rate, in which the cultivar APR ($350,8 \pm 1,59$ $\mu\text{mol/molCO}_2$) had a significantly higher CO₂ exchange rate, when compared to almost all the cultivars in analysis (except for SDL – $351,0,8 \pm 5,09$ $\mu\text{mol/molCO}_2$). SDL also had a significantly higher CO₂ exchange rate, when compared to BUR ($316,9 \pm 13,1$ $\mu\text{mol/molCO}_2$), PDA ($323,9 \pm 8,16$ $\mu\text{mol/molCO}_2$) and WHI ($314,9 \pm 10,2$ $\mu\text{mol/molCO}_2$).

4. Discussion

Catharanthus roseus' huge importance and value, both medicinal and economic, highlights the need to optimize its' alkaloid production. The pharmaceutical market urges in being supplied with higher VBL and VCR quantities at more accessible prices, to answer to the increased demand of oncological treatments, which are prone to continue to rise. Concerning this importance, the statement of the most productive *C. roseus* cultivars would be crucial to overcome the difficulties mentioned above. Therefore, this experiment reveals to be hugely important to understand, among the several *C. roseus* cultivars analysed, which are more prone to be used in massive alkaloid production, due to their morphological and physiological growth and development, and due to their susceptibility to morpho-physiologically respond to the N-UV light treatments.

After the analysis of the obtained results in the "Results" section, and concerning the morphological parameters, a tendency to a significantly smaller production in SDL and APR is observed. These two cultivars have showed to be less productive in several parameters, such as plant height (APR only), number of leaves (SDL and APR), leaf area (SDL and APR) and total dry weight (SDL and APR). On the other hand, PDA, WHI, CDL and TIT revealed a significantly higher vegetative production in several morphological parameters, namely plant height (PDA, WHI, CDL and TIT), number of leaves (PDA, WHI and CDL), leaf area (PDA and CDL) and total dry weight (PDA and WHI)

Regarding plant height, results demonstrated that "cultivar" was the only factor that influenced significantly different results, which showed that APR was significantly smaller than several cultivars in analysis, such as PDA, WHI, TIT, CDL, BUR and PUN. Obviously, being plant height one of the main morphological parameters, because of its' influence on the robustness of plants, these results demonstrate that APR could be a cultivar with higher brittleness, as these results were influenced only by the genetic characteristics of each cultivar. Also, it is important to refer the absence of a significantly different response to the "light quality" factor, which indicates that plant height was not disturbed by the N-UV 2h light quality extension, which is an indicator of an absence of any stress, contrarily to what was observed to happen in experiments with smaller UV wavelengths provided to plants [50].

Number of leaves is one of the most crucial factors for this experiment, as it is where the majority of VBL and VCR metabolic pathway occurs, and it is where they are mainly concentrated [51]. Concerning number of leaves, the parameter "light quality" had a significantly influence, as the N-UV 2h light treatment extension caused a decrease in

leaves production, when compared to the control group. These results seem to indicate a slight activation of a defence mechanism, by decreasing leaves production to save energy and prevent excessive water loss, on plants exposed to the N-UV light spectrum, which could be an indicator of a light stress condition.

Furthermore, and still regarding leaves importance, leaves dry matter also is one of the most important indicators of plants' acclimatation and response to a UV light-induced stress. Also, being dry matter a true indicator of the plants' vegetative growth, and being the main organ in which alkaloids are concentrated, this parameter is one of the key parameters to understand if and how *C. roseus* respond to the N-UV light quality applied in this experiment. Regarding leaves dry matter, results indicate statistically significant differences in "cultivar x light quality" crossing. Here, the "cultivar" factor revealed to be important to distinguish the types of responses of the analysed cultivars to the N-UV light-spectrum. It is possible to observe that some cultivars seem to increase its leaves' dry matter when exposed to N-UV, while others seem to decrease it, although not always with statistically significant results. These different responses are due to the genetic characteristics of each cultivar. Also, it is observable the tendency to SDL plants to be less productive, in this case in their leaves' dry matter, as the control group of this cultivar had statistically inferior leaves DM, when compared to several other groups in analysis, both from the control group (BUR and PDA) and the N-UV group (PUN and WHI). It is possible to refer, acknowledging that DM is one of the best indicators of the real vegetative growth in a plant, that the genetic characteristics of PDA are much more prone to develop bigger and heavier leaves, than SDL, when comparing the control light conditions' results of these two cultivars. Moreover, the "light quality" factor seems to be impacting leaves dry matter, as a tendency is observable to an increase in leaves DM when the N-UV light-spectrum extension is applied (however not observable in every cultivar). This result is an expected one, since it has been observed that UV lights with higher wavelengths (> 315 nm) tend to increase leaves dry matter [50]. Also, and correlating with the already referred decrease in leaves production when exposed to N-UV, the increase in DM could be an indicator of the activation of plants' defence mechanisms, hypothesis that needs to be linked with LA and, upmost, SLA, to be concluded.

Thus, and to properly analyse *C. roseus*' responses to the N-UV light treatment applied in this experiment, LA and SLA must be linked to the already referred increase in leaves dry matter content. Concerning leaf area results, these reveal a significantly difference in the "cultivar" factor, in which PDA and CDL have significantly higher LA than SDL and APR. However, in SLA it was observed a significantly decrease in plants exposed to the

N-UV light spectrum, which allows the conclusion that, since LA did not have any statistically significant results for the “light quality” factor, this decrease in SLA is totally consequence of an increase in leaves’ thickness, which explains the increase in leaves’ DM. These results allow the conclusion that plants could be under a slight stress induced by N-UV, which caused an increased leaves’ thickness (due to the increased dry matter, and to the maintained LA associated with the decreased SLA), associated with a decreased number of leaves, which was a plant’s response to limit transpiration and, thereby, decrease a dehydration risk, as proven to be a plant’s response to an UV-enriched light treatment [50]. Nevertheless, these results indicate that the *C. roseus* cultivars in the experiment did not have a highly negative reaction to the N-UV light quality (when observing their LA) by not being significantly influenced, contrarily to the mainstream plants’ responses to aggressive wavelengths of UV light-spectrums, which tend to diminish their LA, as UV’s stress caused on plants forces them to diminish their water losses and to increase their leaves’ thickness [52].

Concerning root/shoot ratio, as it was observed, results showed no statistically significant alterations, neither between treatments, nor between cultivars. This calculated ratio reinforced the lack of adverse reactions from *C. roseus* to the N-UV light treatment, which maintained its root’s biomass. These results are adequate to the already referred conclusions, in which is understood that the *C. roseus* cultivars in analysis were (although with one or two exceptions) not negatively disturbed by the N-UV light treatment applied. Acknowledging the impact that UV-wavelengths normally have on this parameter, which is characterized by a decrease in roots’ dry weight, mainly by inhibition of their lateral development [53], the obtained results could be considered positive and promising.

Approaching the number of flowers produced, this parameter revealed significantly different results for the “cultivar x light quality” crossing. Here, it is observed a tendency to a higher flower production on the cultivars WHI and TIT, when compared to RED, SDL and APR (the statistically differences between this last cultivar and WHI are even statistically evident). Moreover, it can be observed that the several cultivars reacted differently to the N-UV treatment applied. As it can be observed, TIT plants exposed to an N-UV light treatment had an increase that, although not significant to the TIT’s control group, it is significant to several groups in analysis. Both APR treatments (control and N-UV), both SDL treatments (control and N-UV), both RED treatments (control and N-UV) and PUN (control) are significantly less productive in their number of flowers, when compared to TIT’s N-UV exposed plants. Furthermore, these results are not observed in TIT’s control group (this group is not significantly different from any of the remaining

groups in analysis). Being flowers one of the organs in which the valuable alkaloids CAT, VDL and VBL are found (although not being the main organ of alkaloids concentration) [54], TIT could be considered the most promising cultivar, since it revealed to be positively affected by the N-UV light quality, concerning flower production, which is also evidenced to be accomplished with UV light treatments in other researches [55], fact that could be a relevant result for the screening's final conclusions. Nevertheless, being N-UV wavelengths absorbed by cryptochromes, and being these photoreceptors the precursors of flowering induction [53], it could have been expected a general increase in flower production. However, this increase was not accomplished, meaning that a higher N-UV light intensity could be needed to increase the number of produced flowers.

Acknowledging the fact that CAT, VDL and VBL are also found in flowers, flower dry matter is also an important factor to be considered. Results in this parameter showed that the "cultivar" x "light quality" crossing revealed statistically significant results, meaning that the genetic characteristics of each cultivar determine the type of response that they show to the N-UV light spectrum. However, it is observable that light quality itself did not have an impact on flower DM, as some cultivars showed a tendency to increase their flower DM, while other showed an opposite tendency. These results are in line with the state of the art, in which it has been observed that UV light does not affect flower biomass [56]. Furthermore, it is observable that PDA's and WHI's flowers had a significantly higher DM (although exclusively in the control group), when compared to SDL and APR. These results, associated to the number of produced flowers, shows that SDL and APR are clearly less productive regarding flower production. On the other hand, WHI, PDA and TIT seem to be the cultivars that were more productive.

Still regarding flower production, flowering time is an important parameter to be measured, since it determines the achieving of the plants' full development and, therefore, pinpoints which cultivars develop in a more rapid pattern, which could be relevant in a massive alkaloid production. Results indicate a statistically faster flowering time for the cultivars CDL and TIT, when compared to SDL and APR. Although could have been expected a significantly different result in the "light quality" factor, due to N-UV's role in flower induction [53], there are no significantly different results. So, concerning flowering time, the factor "cultivar" was significantly important, which is result of the different genetic characteristics of each cultivar.

Considering *C. roseus*' physiological parameters, results reveal a higher tendency to an equilibrium between cultivars. SPAD index, which allows a calculation of a relative chlorophyll content, is an example of that, as results indicate no significant results in any of the factors in analysis. Being normally downgraded by smaller wavelength UV light,

due to this light-spectrum's down-regulation of genes that encode chlorophyll's (a and b) binding proteins, chlorophyll is expected to degrade under these range of light-spectrums [53]. However, in the *C. roseus*' cultivars in the experiment, the N-UV light quality to which they were exposed did not cause any significant diminished chlorophyll content, meaning that N-UV was not relevant to chlorophyll synthesis in *C. roseus*, due to the low intensity in which it was provided to plants.

Photosynthetic rate, being the parameter that demonstrate photosynthesis efficiency, is of crucial importance to understand how, physiologically, plants are reacting to the environment. Regarding this parameter, results indicate a significantly higher photosynthetic rate in APR, PUN and TIT, when compared to WHI and PDA. APR has showed to have a higher photosynthetic rate even when compared to SDL and BUR. However, these differential in results are exclusively due to the "cultivar" factor, as the "light quality" factor did not have statistically significant results. N-UV light is reported to influence photosynthetic rate, due to the signalling that phototropins, which absorb N-UV light, induce in plant's physiological mechanisms, such as stomatal opening (which allows an uptake of CO₂) and chloroplast movement [53], that allow plants to optimize photosynthesis. However, this N-UV light's influence was not verified in results, fact that could be linked with a low intensity of the provided UV-light treatment.

Approaching the aims of this experiment, and considering the screening that was proposed to perform, these results, which significantly differences are mostly associated to the "cultivar" factor (as observed in table 1), clearly indicate a higher morphological production of PDA, WHI, CDL, and TIT, when compared to SDL and APR, differences that result from the genetic differences between the cultivars. The huge majority of the morphological parameters in analysis proposes that the cultivars APR and SDL are statistically inferior to most of the remaining ones, allowing to state that these two cultivars are not suitable for a possible industrialization of alkaloid production by *C. roseus*.

TABLE 1: SYNTHETIZATION OF THE INFLUENCE OF THE DIFFERENT PARAMETERS ANALYSED. GREEN BOX MEANS A SIGNIFICANT INFLUENCE BY THE FACTOR IN THE CORRESPONDENT PARAMETER; RED BOX MEANS NA ABSENCE OF A SIGNIFICANT INFLUENCE BY THE FACTOR ON THE CORRESPONDENT PARAMETER

	CULTIVAR X N-UV	N-UV	CULTIVAR
HEIGHT			
NUMBER OF LEAVES			
LEAF AREA			
SPECIFIC LEAF AREA			
TOTAL DRY MATTER			
LEAF DRY MATTER			
FLOWER DRY MATTER			
ROOT/SHOOT RATIO			
NUMBER OF PRODUCED FLOWERS			
FLOWERING TIME			
RELATIVE CHLOROPHYLL CONTENT			
PHOTOSYNTHETIC RATE			
GAS EXCHANGE RATE			

Regarding the most productive cultivars, WHI, PDA, TIT and CDL were, by far, the most productive cultivars in almost every morphological parameter analysed in the experiment (as observed in table 2). Analysing leaves production, which is the main organ to be considered to alkaloid production and extraction, PDA was the only cultivar with significant results in all three parameters that had relevant results (number of leaves, LA and leaves DM), which may allow the conclusion that, regarding leaf production, it is the most productive one. Regarding flower production, these four cultivars were substantially more equivalent, although the parameter “flowering time”, which has a substantial importance in a possible massive production to alkaloid extraction, showed a significantly faster flowering to TIT and CDL.

However, regarding the photosynthetic rate’s results, PDA and WHI are the least photosynthetically active cultivars. As these results are only significant in the “cultivar” factor, it can be concluded that these handicapped photosynthetic rates are due to these cultivars’ genetic characteristics, which can be limiting biomolecules synthesis, such as CAT, VDL, VBL and VCR. Moreover, these conclusions are reinforced by the results obtained in the measured gas exchange rates, where it is possible to observe that the amount of consumed CO₂ by WHI and PDA is significantly lower than SDL and APR. However, these cultivars should be carried into further analysis.

TABLE 2: SCREENING RESULTS AND CORRESPONDENT BEHAVIOUR OF THE DIFFERENT CULTIVARS, CONCERNING THE REFERRED PARAMETERS

	SCREENING	
	+	-
HEIGHT	TIT, CDL, WHI, BUR, PDA	APR
NUMBER OF LEAVES	CDL, BUR, WHI, PDA	SDL, APR
LEAF AREA	CDL, PDA	SDL, APR
SPECIFIC LEAF AREA		
TOTAL DRY MATTER	BUR, WHI, PDA	SDL, APR
LEAF DRY MATTER	CDL, BUR, PUN, WHI, PDA	SDL
FLOWERS DRY MATTER	WHI, PDA	SDL, APR
ROOT/SHOOT RATIO		
NUMBER OF PRODUCED FLOWERS	TIT, WHI, PDA	SDL, APR
FLOWERING TIME	TIT, CDL	BUR, SDL, RED, PUN, APR, PDA
RELATIVE CHLOROPHYLL CONTENT		
PHOTOSYNTHETIC RATE	TIT, PUN, APR	WHI, PDA
GAS EXCHANGE RATE	SDL, APR	WHI, PDA

Resembling the influence that the N-UV light-spectrum had on the experiment, it had a significantly influence in the number of leaves, decreasing its number. Furthermore, SLA were also influenced by the N-UV light treatment but, in this case, it enhanced leaves thickness. These results allow the conclusion that N-UV light spectrum had a slight impact in *C. roseus* plants, mainly in the increase in leaves' thickness and DM, which can be an indicator of a defensive reaction to the applied N-UV. However, *C. roseus* plants seem to be reacting positively to the N-UV light spectrum, statement that can be reinforced by the absence of a statistically alteration in the root/shoot ratio, and by the enhancement of one of the main parameters to be considered in alkaloid production, leaves DM. As N-UV light stimulates flowering (by being absorbed by CRY), and stomatal and chloroplast motion (by being absorbed by phototropins), and regarding the good adaptation showed by *C. roseus* to the N-UV light, it would have been expected a "light quality" factor's influence and a consequent increase in flower production, flowering time, photosynthetic rate and gas exchange rate. This general lack of influence of the N-UV light spectrum could be, in one hand, considered to be positive, meaning that the treatment applied did not cause any severe stress in the experimented cultivars. With this conclusion it is possible to affirm that this light quality can be applied to test these cultivars for their alkaloid content, without having any highly negative morphological or physiological effects on plants. However, as N-UV is related to enhance flower production, flowering time, photosynthetic rate and gas exchange rate, the absence of statistically different results in these parameters, which would result of the influence of the N-UV light quality provided, could be motivated by a low intensity light applied to

plants. A higher intensity N-UV light spectrum would, possibly, increase these parameters' production and, consequently, be more prone to be hypothesized and tested for an increase in alkaloid production. Also, it is possible that the 2h extended period, in which the N-UV light treatment was provided, was not sufficient to enhance morpho-physiological production.

5. Conclusions

With this experiment, and concerning the objectives to which it was proposed, it was possible to screen several *C. roseus* cultivars, under two light qualities (R+B and R+B+N-UV), and to pinpoint some of them, regarding their morpho-physiological production. Among the 9 *C. roseus* cultivars in the experiment, TIT, CDL, WHI and PDA were, in general, the most productive ones and should be selected to be analysed concerning their alkaloid content. Although WHI and PDA had a low photosynthetic and gas exchange rates, their morphological production was the highest in almost every morphological parameter, which justifies this selection. On the other hand, SDL and APR showed to be the least productive cultivars, concerning their morphological parameters, and should be discarded for an industrial alkaloid production.

Regarding N-UV light spectrum's impact on *C. roseus*, globally it was not highly harmful to the cultivars' normal growth and development, although causing a diminished number of leaves and an increase in leaves DM and thickness. Despite this, it can be declared that there is an absence of a big impact on the enhancement of the morpho-physiological parameters in analysis, despite what could have been expected. Furthermore, it is possible that a perfected N-UV light quality (with higher intensity and/or longer period of provisioning) enhanced significantly the morpho-physiological production in the experimented plants. Nevertheless, N-UV light has showed to influence leaves DM, which could be a promising result.

6. Future works

Concluding the screening proposed in this experiment, and regarding the obtained results, future perspectives should englobe the analysis of the alkaloid content, gene expression and protein quantification should be performed, so it can be recognized the productive potential of each cultivar, which may be important to select or discard any cultivar.

Furthermore, it would also be important to understand the impact of conditions such as temperature and relative humidity to both morpho-physiology and alkaloid production, in the cultivars that had a higher morpho-physiological production (TIT, CDL, WHI and PDA). It is important that all environmental conditions are optimized, in order to maximize this production.

Finally, considering the general lack of impact of the N-UV light quality, an increase in this wavelength's light intensity should be considered, as well as an increase in the period of exposure of plants to the N-UV wavelength.

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