DEPARTMENT OF AGRICULTURAL SCIENCES
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**Ph.D. Thesis in Sustainable Agricultural and Forestry Systems and Food Security**

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# The role of pollen in Plant resilience to climate change

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*“Earth provides enough to satisfy every man's needs, but not every man's greed.”*

Mahatma Gandhi

# Preface

The work presented in this thesis was carried out in the period from December 2018 to December 2021 at the Department of Agricultural Sciences of the University of Naples Federico II. The supervisor was Prof. Giovanna Aronne. A period of two months was spent working with Prof. Lilach Hadany in the Department of Molecular Biology and Ecology of Plants at the University of Tel-Aviv (IL). Another period of activities abroad was conducted in the Netherlands, at the European Space Research and Technology Centre (ESTEC) of the European Space Agency (ESA). The Ph.D. project was funded by the University of Naples Federico II.

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# Introduction

## General context

Plant reproduction is the only natural process able to improve genetic variability of the offspring and enhance the probability of obtaining new genotypes better adapted to survive in a changing environment. Rare and endangered plants are considered less able to adapt to changing environmental conditions because their populations lack the necessary genetic variability to survive and reproduce (Spielman et al., 2004). Besides rare plants, most of crop species require the full completion of all the phases occurring within their reproductive cycle to produce seeds and/or fruits. Further, most of the plant species currently studied for cultivation in space are grown to produce leafy crops starting from seeds produced on ground even though long term space mission will require the full completion of seed-to-seed cycles on board spacecraft.

In this framework, researchers on reproductive biology within the research topics reported above, should follow a bottom-up approach aimed at investigating vulnerability of each stage of the reproductive cycle to environmental constraints in order to focus their studies only on reproductive traits that limit reproductive fitness (Aronne, 2017).

Pollen interaction with the environmental factors represents a potential bottleneck that can drastically limit reproductive success both in rare and in crop species. Along its journey from the stamen to stigma landing, pollen needs to stay viable and germinable to trigger double fertilization.

Among environmental constraints, high temperatures and humidity are well known to affect pollen viability, pollen germinability and fertilization success (Pérez et al. 2019; Aronne et al. 2015). In rare and endangered species, high temperature combined with high humidity can affect pollen viability leading to disruption of crucial reproductive traits such as dichogamy, heterostyly and self-incompatibility that resulted evolutionary successful to enhance outcrossing and increase genetic diversity of the offspring (Cardoso et al., 2018).

Pollen sterility induced by high temperature stress is crucial in agricultural systems because it limits the productivity of many crop species (Pacini and Dolferus 2019). Negative effects of high temperature on pollen functionality constitute possible

bottlenecks in plant life cycle of many species (De Micco et al. 2014; Aronne 2017). Therefore, a deep understanding on how high temperatures affect pollen functionality is essential to ensure food security. At present, a special need to deepen pollen reaction to altered temperature conditions is relevant not only to predict the effect of gradual global warming on crop productions, but also to prevent the effect of sudden changes in temperatures during single producing cycles. Indeed, frequencies of heat waves is increasing and impacting cultivations both in open field and in greenhouses and might strongly affect quantity and quality of crop production (Ledesma and Kawabata 2016). Previous studies on the effect of high temperature at flowering, generally focus their attention on the functionality of the male gametophytes and are performed incubating mature pollen grains samples after their release from the anthers. However, negative effects of high temperature before pollen development have been described on microsporogenesis of several species (Porch and Jahn 2001; Szalay et al. 2019; Masoomi-Aladizgeh et al. 2020). Among crops, a considerable number of studies has been conducted to assess heat stress on tomato pollen, because of its agronomic relevance and its exposure to temperature fluctuations during flowering, both in open field and in greenhouse. In tomato heat stress is also reported interfere with tapetum formation, exine deposition and vacuolization of mature microspores (Giorno et al. 2013) and also with starch accumulation (Pressman et al. 2002). These heat stress-induced defects are produced during the microsporogenesis, may strongly affect the progression of male gametogenesis, and therefore the proper formation and functionality of mature pollen. Despite the general knowledge on the effect of temperature on plant reproduction, studies aimed at investigating possible implications of heat stress-induced aberrations during the later gametogenesis phases have been generally neglected.

Also humidity combined with different temperatures exposure can affect pollen functionality throughout pollination (Aronne 1999). More specifically, humidity would enhance the negative effect of high temperature on pollen longevity influencing pollen hydration state. Water content in pollen grains generally decrease after flower anthesis and anthers dehiscence once that pollen is exposed to the environment. From anthesis on, low humidity exposure can induce pollen into a quiescent state

of development and resist to better resist heat. Particularly, in cross-pollinated species, pollen needs to survive for a relative long time, and therefore, it needs to reduce its water content to maintain a metabolically inactive state during its journey to the stigma of other flowers. However, this quiescent state can be altered by high humidity exposure reducing capability of pollen to conserve its functionality over time.

To have a complete overview of pollen response toward environmental constraints, both pollen viability and germination should be tracked throughout the period from pollen dispersion till pollen germination onto the stigma. Both pollen viability and germination represent crucial reproductive traits characterising pollen functionality: viability tests evaluate the effective fulfilment of the pollen grain development process; in vitro-germination tests measure the capability of pollen to elongate a pollen tube and potentially succeed in double fertilization.

More in details, pollen viability and germination should be assessed in the course of the effective pollination period (EPP), defined as the number of days during which pollination is effective in fruit set because pollen functionality, stigmatic receptivity and ovule longevity overlap (Sanzol and Herrero, 2001). Therefore, studies that simulate EPP performing pre-incubation treatments on pollen before in-vitro germination, might better evaluate reproductive fitness both in crops and rare species.

## General aim of the thesis

The aim of the thesis was the identification of the main bottlenecks occurring in reproductive cycle of rare and crops species. In this regard, an in-depth review of literature review regarding the effect of environmental factors on the reproductive processes of angiosperms was carried out in the first part of the project. In the framework of reproductive ecology, pollen response to environmental constraints was often reported as a limiting factor for crops productivity and rare species conservation. A more in-depth follow-up investigation throughout pollen ontogenesis highlighted a different pollen sensitivity depending on its developmental stage. Literature survey confirmed that despite many heat stress-induced defects were

reported in microsporogenesis, specific studies investigating possible relations between a heat stress occurring during the microsporogenesis and a later implication on the gametogenesis were neglected.

All research activities carried out during the Ph.D. aimed to bridge this gap of knowledge deepening the effect of the main factors affecting pollen functionality.

This approach was applied both to wild species threatened with extinction and to crop species. In the first case the main aim was to contribute at identifying the bottleneck limiting the generation turnover and the long-term conservation of the species (Chapter 1). For crops the main goal was to analyse the species resilience to environmental factors in a scenario of climate changes (Chapters 2-4). Additionally to these main aims of the thesis, the approach of analysing the interaction between plants and environmental factors was also extended to space factors, and more specifically to altered gravity conditions. These research activities were aimed at contributing to succeed in the long-term mission to Moon and Mars by using plants in Bioregenerative Life Support Systems (*Appendix 1*).

## Specific objective of the thesis

The thesis consists of four Chapters and three Appendixes. Their content is summarized hereby.

*Chapter 1* synthetize the experiments on the effect of three different temperatures and three levels of relative humidity on pollen viability of both short-styled and long styled flowers of *P. palinuri* Petagna, a relict chasmophyte classified as Vulnerable due to its decreasing populations. Results on this Primula species suggest that in a scenario of climate changes, high temperature and humidity exposure can differently affect pollen functionality of the two flower morphs and may lead to disruption of distyly equilibrium. Results of this experiments are reported as in the article published on *Annali di Botanica* (Aronne et al., 2019).

In *Chapter 2* results of several experiments on the effects of high temperatures during pollen microsporogenesis of *Solanum lycopersicum* L. are reported. The experiment was performed on the ‘Micro-Tom’ cultivar and shows how pollen sensitivity changes according to pollen developmental stage targeted by heat

treatment. Particularly, results unravel that heat treatment restricted to earliest stages of pollen development is responsible for a drastic loss of pollen viability and germination of pollen once ready for dispersal, and reduction of its lifespan. Data are reported as in the article published on *Plant Reproduction* (Iovane et al., 2021).
In *Chapter 3* is reported the key role of humidity in determining pollen thermo-tolerance in *Olea europaea* L. Results highlighted that in the high RH treatment pollen viability significantly decrease over time at any temperature condition, whereas at low RH enhanced pollen thermo-tolerance to both low and high temperature and preserved pollen viability over time. An article reporting this study has been published in *Agronomy* (Iovane et al., 2022).
*Chapter 4* shows differences in pollen resilience of 13 apricot cultivars traditionally cultivated in Southern Italy. Despite high pollen viability was assessed in all the cultivars at anthesis, pollen germination evaluated within the effective pollination period of *Prunus armeniaca* showed a consistent variation according to temperature exposure of pollen during incubation, except for two cultivars that stood out for their pollen resilience to low and high temperatures. Results of this experiments are reported as in the manuscript submitted to *Scientia Horticulturae.*
*Appendix 1* reports the results of the experiment performed in European Space Research and Technology Centre (ESTEC) as team member in ROOTROPS project whose objective is to evaluate the variations in tropic effects of different light wavelengths and light intensity in orienting root growth under hyper gravity and simulated microgravity conditions. Results of ROOTROPS experiment are showed in *appendix 1A* and *1B* in two published articles (Izzo et., al 2021; Aronne et al 2021) in which I am co-author.
*Appendix 2* describes the two research activities conducted abroad. More specifically, it refers to the period of three weeks in The Netherlands, at the European Space Research and Technology Centre (ESTEC) of the European Space Agency (ESA), and to that of two months abroad at the University of Tel-Aviv (IL).
*Appendix 3* includes a brief summary of all the other activities conducted during the Ph.D. including seminars, conferences, oral presentations, and articles published.

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# Chapter 1

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## TEMPERATURE AND HUMIDITY AFFECT POLLEN VIABILITY AND MAY TRIGGER DISTYLY DISRUPTION IN THREATENED SPECIES

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# Temperature and humidity affect pollen viability and may trigger distyly disruption in threatened species

## 1.1. Abstract

Within the ongoing trend of rapid climate changes there is an urgent need to assess the vulnerability of the endangered species for evaluating their conservation status and planning effective actions.

*Primula palinuri* Petagna is a relict chasmophyte classified as Vulnerable due to its decreasing populations. The species evolved a distylous syndrome: a reproductive system that maintains a high genetic variability by enhancing cross-pollination.

Using an experimental approach, we compared the effect of three different temperatures and three levels of relative humidity on pollen viability of both short-styled and long styled flowers of *P. palinuri*.

Data highlighted that temperature and humidity significantly affect pollen viability. Notably, pollen of short-styled flowers showed a significant higher viability than the long-styled morph in any of the tested treatments. Such results raised the concern for distyly equilibrium disruption in this species, a phenomenon that in isolated populations may cause a sharp raising of the risk of local extinction.

Overall, results on this *Primula* species suggest that in a scenario of climate changes the effect of environmental factors on pollen functionality of any endangered distylous species should not be overlooked for their vulnerability assessment and conservation status evaluation.

## 1.2. Introduction

It is well known that climate changes can drastically increase the risk of extinction of many species presently surviving in restricted geographical areas (Brook et al., 2008). Such a risk is enhanced when their generation turnover is limited by the occurrence of bottlenecks in the fulfilment of the reproductive cycle and production of new individuals (Aronne, 2017). At species level, reproductive traits as dichogamy, heterostyly and self-incompatibility resulted evolutionary successful to enhance outcrossing and increase genetic diversity of the offspring (Cardoso et al., 2018). However, within the scenario of rapid climate changes such traits can result as a constraint to achieve successful reproduction because of the complex interactions with environmental factors.

Distyly is the most common type of heterostyly and is characterized by individuals presenting two floral morphs, which reciprocally differ in the heights of anthers and stigmas within flowers; consequently, in the same population there are plants with flowers with long styles and short stamens (long-styled or pin morph), and others with short styles and long stamens (short-styled or thrum morph) (Cardoso et al., 2018). Distyly was reported at first by Darwin (1877) in *Primula* species and thereafter reported in 26 families (Naiki, 2012). The hypothesis that heterostyly evolved to promote outcrossing has been widely studied and verified (e.g., Barrett, 1992; Lloyd & Webb, 1992; Stone & Thomson, 1994).

Species of the *Primula* genus represent the classic study model for in-depth genetic studies of heterostyly syndrome (e.g. Mather, 1950; Guggisberg et al., 2006; Naiki, 2012). In Europe, *Primula* species belonging to the sect. Auricola diversified under the selective pressures of the climate conditions of the Pleistocene and more than 20 species are endemic to restricted areas of high mountains (Zhang & Kadereit, 2004). Throughout the Pleistocene, cold periods caused the migration of the populations to lower altitudes and latitudes and warm phases caused range contraction, local extinction, and consequent genetic isolation (Zhang & Kadereit, 2004; Crema et al., 2013). At present, all species of the sect. Auricola live in mountain habitats (Zhang & Kadereit, 2004) with the exception of *Primula palinuri* Petagna which is the only Mediterranean and maritime species in the whole genus (Richards, 2003). *P. palinuri* is an endemic species and has a small fragmented

geographical range confined to a narrow belt of the Tyrrhenian coast of southern Italy (Pizzolongo, 1963; Ricciardi, 1973; Aronne et al., 2014). It is a relict chasmophyte reported in the IUCN (International Union for Conservation of Nature) Red List as Vulnerable (VU) species with decreasing population (Rossi et al., 2013). The main threats to the species are human impact, fire, competition with invasive alien species and landslides (Uzunov et al. 2008). Most of the plants of *P. palinuri* live in soil pockets inside rock fractures, on vertical or sub-vertical north-facing limestone cliffs, most of which are sea plunging (Aronne et al., 2014; Strumia et al., 2020)

Identification of the most critical phases in lifecycle of *P. palinuri* pointed to seed germination and seedling establishment as bottlenecks in the turnover of subsequent generations (Aronne et al., 2010; Aronne, 2017). Moreover, specific studies highlighted that seeds and seedlings of *P. palinuri* are less adapted to salted conditions of the sea cliffs than other cliff species co-occurring in the same habitat (Strumia et al., 2020).

Studies on pre-dispersal biology showed that the reproductive success of *P. palinuri* is high: plants flower every year and produce hundreds of seeds. Data on floral morphology proved the occurrence of the fully distylous syndrome: the reciprocal position of stigma and anthers in the flower as well as the intra-morph incompatibility and the necessity of crosspollination between the two morphs to succeed in seed development (Aronne et al., 2013). Plants have an overall flowering period starting in winter (end of January) and ending in spring (mid of April) and single flowers show an extraordinary long anthesis of over a month (Aronne et al., 2014). The long flowering period and the flower longevity have been interpreted as results of two contrasting selective pressures exerted by progressive climate warming: the first bounds the anthesis to Winter temperatures by pollen functional traits; the second extends the flowering season through Spring by the pollination rate increase (Aronne et al., 2014). Previous studies highlighted that pollen viability is significantly lowered by increasing temperature and air humidity, envisaging a rising risk of extinction in the ongoing trend of climate changes (Aronne et al., 2014). The distyly syndrome in *P. palinuri* is not limited to the reciprocal position of anthers and stigmas but is complemented by differences in pollen size and exine ornamentation (Aronne

et al., 2013). However, data on possible different effects of environmental factors on pollen functionality of the two flower morphs are not available.

Considering that in isolated populations any stochastic event altering the equal proportion of the two morphs in favour of a single genet can cause a sharp raising of the risk of local extinction (Endels et al., 2002; Kéry et al., 2003; Brys et al., 2004), we compared pollen functionality of long-styled and short-styled flowers of *P palinuri* under different combinations of environmental factors. More specifically, using an experimental approach we assessed the effects of different temperatures and humidity values on pollen viability of the two morphs for a time period comparable to flower anthesis.

### 1.3. Material and Methods

Plants of *Primula palinuri* Petagna mainly occur on ledges and crevices of north-facing vertical cliffs along a narrow coastal area of about 65 km long and 1 km wide between Palinuro (40°02'04"N - 15°17'08"E) and Capo Scalea (39°50'47"N - 15°46'44"E).

In the field, we harvested a total of 30 pendulous flowers whose phenological stage corresponded to beginning of the anthesis. More specifically, for each of the two floral morphs we sampled one flower per plant, from five plants, from three different populations. Flowers were individually labelled and immediately transferred to the laboratory. Here, we collected and carefully mixed the pollen from all the flowers obtaining a bulked pollen sample for each of the two flower morphs.

We experimentally tested the effects of temperature, relative humidity (RH) and time on pollen functionality. Pollen functionality was assessed as pollen viability and measured by means of the diaminobenzidine (DAB) reaction (Dafni et al., 2005).

The choice of the environmental parameters followed the criteria reported by Aronne et al. (2014). More specifically, we used three different treatments of RH (0, 48 and 100%) and temperature (6, 18 and 30°C). Different conditions of RH were obtained in a sealed glass chamber (20x20x20 cm) according to Aronne et al. (2006). In particular, the condition of 100% RH was obtained by lining the chamber with moist filter paper, that of 48% RH with a saturated water solution of NaI and that of 0% RH

with silica gel. The experiment was performed according to a factorial design in which humidity and temperature conditions were cross linked. Overall, we used 2 flower morphs x 3 temperatures x 3 humidity values x 4 time periods. Per each treatment we prepared 3 slides and scored a minimum of 250 grains per slide. Pollen was spread onto distinct microscope slides, placed into the sealed glass chambers, and transferred in three incubators set at different temperatures (VELP®, FOC 200IL). We checked pollen viability at different time intervals that resembled the long anthesis of a single flower of *P. palinuri*. More specifically, after 1, 11, 20 and 32 days, one slide for each of the nine combinations of temperature and humidity was taken out and scored for pollen viability.

At the end of each treatment, to assess pollen viability we added one droplet of DAB solution on each pollen sample, gently warmed the slides with a gas lighter, waited about one minute for staining reaction, and mounted with a cover slip (Dafni, 1992). We scored the samples using an Olympus® BX-60 light microscope and classified as viable only the dark-brown grains.

*Statistical analysis* - Descriptive statistics were used to calculate central tendency, dispersion and distribution of data. To apply parametric inferential statistic, the symmetry of data was checked; in case of asymmetry, the logarithmic transformation was performed (Sokal & Rohlf, 1995). The effect of each of the independent variables (temperature, RH and time) was tested separately using one-way ANOVA. If a statistical difference between treatments resulted from the one-way ANOVA, the post-hoc multiple comparison analysis (Bonferroni $P<0.05$) was performed.

### 1.4. Results

Overall data on the effects of temperature and humidity on the pollen of the two flower morphs showed no morphological abnormalities and uniformly reacted to the DAB enzymatic staining. During the scoring, only few doubtful cases were found and excluded from the count. In the whole experiment, we classified as viable or not viable more than 54000 single pollen grains).

Results of the ANOVA showed that viability gradually decreased during the 32 days long period. Differences in pollen viability resulted highly significant (F-ratio= 7.31, P<0.001 for short-styled and F-ratio = 4.64, P<0.001 for long-styled) for pollen of both flower morphs (Fig. 1).

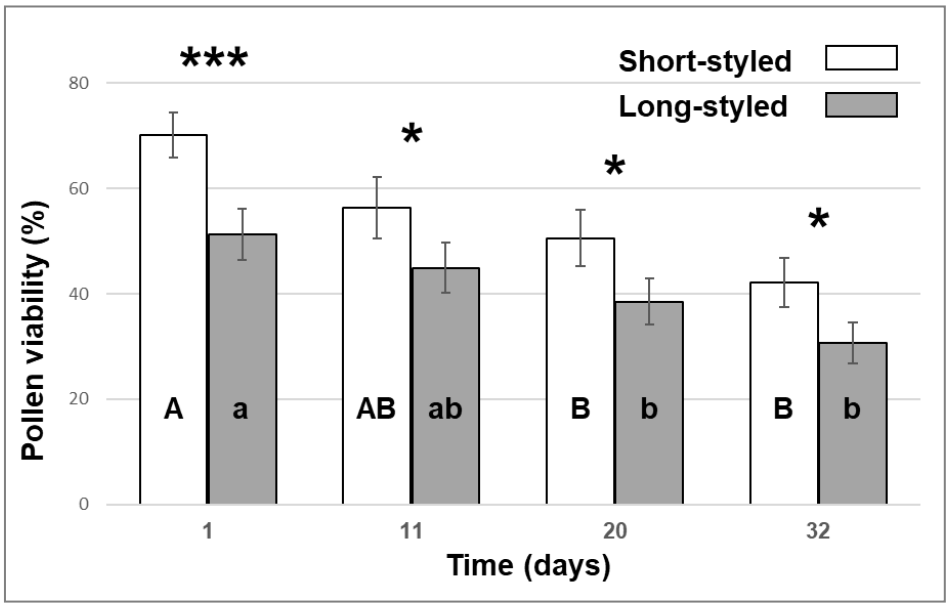


**Fig. 1**. Pollen viability (mean%±SE) of short-styled (white bars) and long-styled (grey bars) flowers at different time intervals (1, 11, 20, 32 days). Results of one-way ANOVA to verify the difference between short- vs long-styled flowers at the same time are reported above each couple of bars (*=P<0.05; ***=P<0.001). Letters (either capital and lowercase) represent the result of the post-hoc test (Bonferroni, P<0.05) of the one-way ANOVA performed to compare pollen viability at different time within each flower morph; times with the same letters are not statistically different.

Considering the effect of RH, the ANOVA main effect was significant (F-ratio= 383.67, P<0.001 for short-styled and F-ratio= 325.45, P<0.001 for long-styled). However, the post hoc analysis showed that in both morphs, viability did not change when pollen was kept at 0 % RH and 48% RH, while it drastically decreased when grains were kept at 100% RH (Fig. 2).

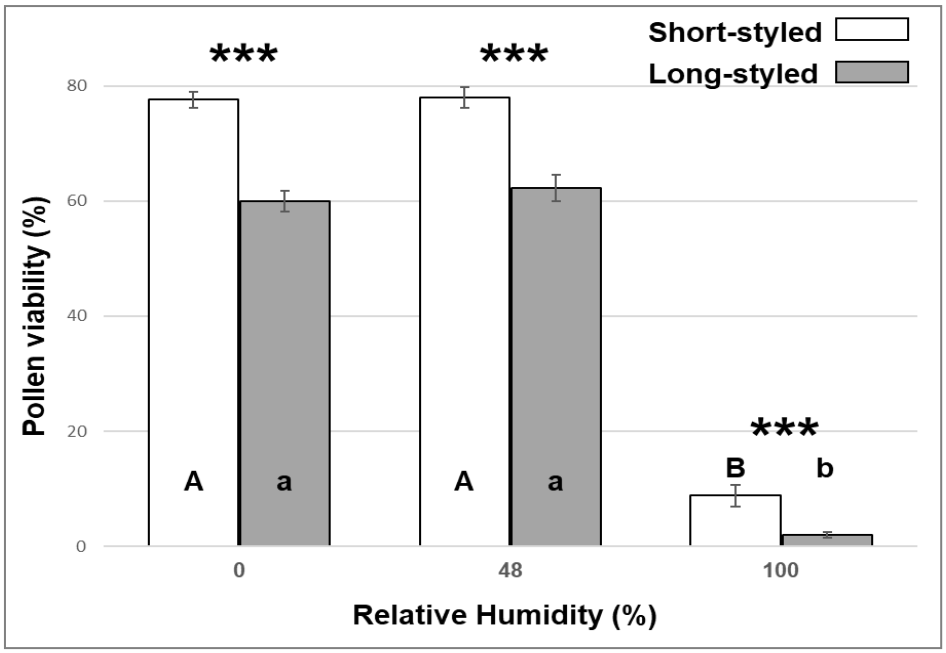


**Fig. 2**. Pollen viability (mean%±SE) of short-styled (white bars) and long-styled (grey bars) flowers at different RH (0, 48 and 100%). Results of one-way ANOVA to verify the difference between short- vs long-styled flowers at the same RH are reported above each couple of bars (***=P<0.001). Letters (either capital and lowercase) represent the result of the post-hoc test (Bonferroni, P<0.05) of the one-way ANOVA performed to compare pollen viability at different RH within each flower morph; RH values with the same letters are not statistically different.

Moreover, comparing viability in each of the three humidity treatments, viability of pollen of short-styled flowers was always significantly higher than that of pollen of long-styled flowers (Fig. 2).

Pollen viability was less affected by temperature (Fig. 3): the ANOVA main effect was significant only for pollen from long-styled flowers (F-ratio= 5.56, p<0.001). In such cases viability significantly decreased (down to 31.4%) only when pollen was incubated at 30°C. In addition, considering each temperature treatment, differences between the morphs were always significant with pollen of short-styled flowers being more viable than that of long-styled flowers (Fig. 3).

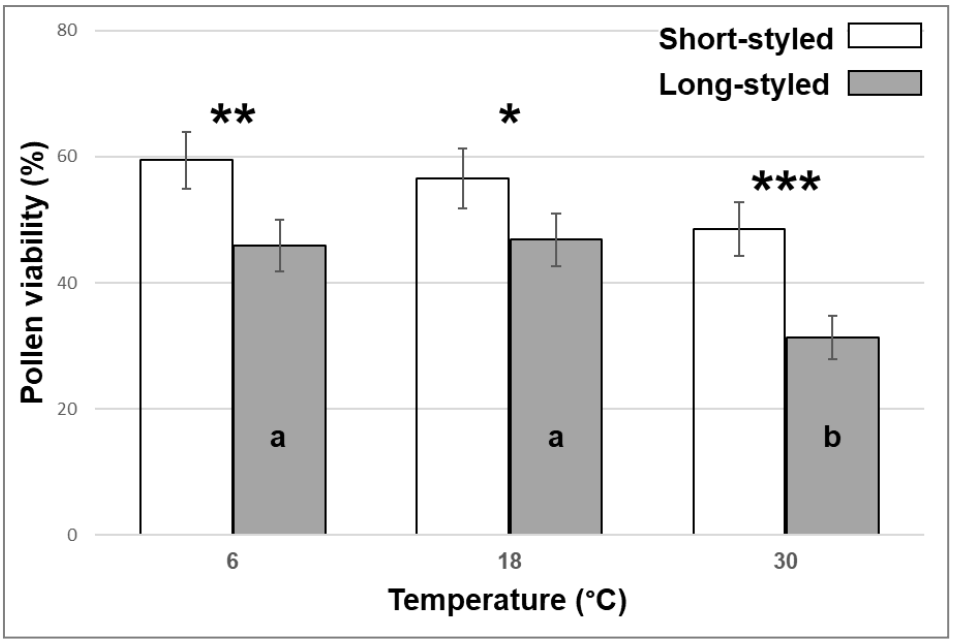


**Fig. 3**. Pollen viability (mean%±SE) of short-styled (white bars) and long-styled (grey bars) flowers at different Temperature (6, 18 and 30°C). Results of one-way ANOVA to verify the difference between short- vs long-styled flowers at the same Temperature are reported above each couple of bars (*=P<0.05; **=P<0.01; ***=P<0.001). Letters represent the result of the post-hoc test (Bonferroni, P<0.05) of the one-way ANOVA performed to compare pollen viability at different Temperature within each flower morph; Temperature values with the same letters are not statistically different.

## 1.5. Discussion

Single pollen grains are autonomous, haploid and genetically different organisms constituted only by two (max three) incorporated cells; they must survive and interact with the environment throughout the period from pollen release to pollen transfer onto the stigma and subsequent pollen tube development. During plant reproduction, pollen is thus exposed to natural selection and can influence the genetic constitution of the resulting diploid sporophytic generation (Mulcahy, 1979). Our data showed that pollen of *P. palinuri* is affected by both high temperature and humidity confirming results reported in a previous study (Aronne et al., 2013). Similar effects of temperature and humidity on pollen functionality were reported for other Mediterranean coastal species (Aronne, 1999). Our results further highlight

that pollen functionality should also be taken into account in the overall evaluation of the future conservation status of this endemic species. This is particularly relevant considering the ongoing climate changes, in which the gradual increase of the temperature is complemented by other environmental changes and by the increase of both intensity and frequency of extreme weather events, including heat waves (Hedhly, 2011).

The most significant contribution of our results refers to the differences in pollen functionality between the two flower morphs. Distyly has polyphyletic origins (Naiki, 2012) and is considered a trait that enhances intermorph cross-pollination and genetic variability (e.g., Lloyd & Webb 1992; Stone & Thomson, 1994; Barrett, 2002). As for most of the distylous species, also in *P. palinuri*, in addition to the reciprocal position of anthers and stigma, different pollen sizes and exine ornamentations characterize the two morphs (Aronne et al., 2013). Our data highlighted that pollen of the two morphs interacts differently with the environmental factors raising the concern for the risk of a distyly equilibrium disruption.

Populations of distylous species at equilibrium have short-styled flowers and long-styled flowers equally represented; however, deviation of morph frequencies from 1:1 ratio (anisoplethy) has been frequently reported in distylous species (Kéry et al., 2003). They are generally associated to a) demographic stochastic processes (Kéry et al., 2003); b) morph-specific differences in mating patterns and self-compatibility levels (Barrett & Husband, 1990); c) morph-specific selection in favour or against one morph type (Kéry et al., 2003).

Unequal frequencies of morphs were reported for *Primula* species living in isolated populations and fragmented habitats (e.g. Endels et al., 2002; Jacquemyn et al., 2002; Brys et al., 2003). In such contexts, biased morph ratios can disrupt pollen processes contributing to reduced reproductive success (Matsumura & Washitani, 2000; Brys et al., 2004). Morph anisoplethy can exert negative demographic and genetic consequences for the populations and consequently increase the extinction risk of small populations (Washitani, 1996; Jacquemyn et al., 2003; Kéry et al., 2003).

A fully distyly syndrome has been reported for *P. palinuri* (Aronne et al., 2013). However, a slight (although statistically not significant) prevalence of short-styled

inflorescences was highlighted in all checked populations (Aronne et al., 2013). Our data on pollen functionality clearly showed that pollen from short-styled flowers performed better than that from long-styled flowers in all treatments of temperature and humidity and throughout the whole anthesis period. If such a prevalence will persist in time, a scenario of significant skewed morph ratios in the few isolated populations can lead to a loss of population genetic diversity by increasing the frequency of the most fertile short-styled morph.

Even if *P. palinuri* is currently classified as Vulnerable (Rossi et al., 2013) the decreasing trend of the population is well known and the risk of extinction of local populations may rise sharply, especially considering the effects of climate change.

Skewed ratios in favour of long-styled morph was reported in fragmented populations of *Primula veris*, but no significant relationship was found between genetic patterns and stochastic morph bias (Van Rossum & Triest, 2006); such results raised the hypothesis that individual longevity of the plants in the population and clonality can slow down the expression of the biased morph frequencies. The two morphs are rather balanced also in the populations of *P. palinuri* (Aronne et al., 2010; De Micco & Aronne, 2012; Strumia et al., 2020); however, , our results suggest that in this species differences in pollen functionality can favour one of the two morphs in isolated populations. Considering that pollen is highly and finely affected by the interaction with environmental factors, in a scenario of climate changes the role of pollen for long term survival of distylous species should be not overlooked.

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# Chapter 2

Plant Reproduction
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ORIGINAL ARTICLE


## High temperatures during microsporogenesis fatally shorten pollen lifespan

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# High temperatures during microsporogenesis fatally shorten pollen lifespan

## 2.1. Abstract

Many crop species are cultivated to produce seeds and/or fruits and therefore need reproductive success to occur. Previous studies proved that high temperature on mature pollen at anther dehiscence reduce viability and germinability therefore decreasing crop productivity. We hypothesized that high temperature might affect pollen functionality even if the heat treatment is exerted only during the microsporogenesis. Experimental data on *Solanum lycopersicum* 'Micro-Tom' confirmed our hypothesis.

Microsporogenesis successfully occurred at both high (30°C) and optimal (22°C) temperature. After the anthesis, viability and germinability of the pollen developed at optimal temperature gradually decreased and the reduction was slightly higher when pollen was incubated at 30°C. Conversely, temperature effect was eagerly enhanced in pollen developed at high temperature. In this case, a drastic reduction of viability and a drop-off to zero of germinability occurred not only when pollen was incubated at 30°C but also at 22°C.

Further ontogenetic analyses disclosed that high temperature significantly speeded-up the microsporogenesis and the early microgametogenesis (from vacuolated stage to bi-cellular pollen); therefore, gametophytes result already senescent at flower anthesis.

Our work contributes to unravel the effects of heat stress on pollen revealing that high temperature conditions during microsporogenesis prime a fatal shortening of the male gametophyte lifespan.

## 2.2. Introduction

Successful interaction of pollen with the environment is essential in agricultural systems because most crop species are cultivated to produce seeds and/or fruits and therefore need fertilization to occur. Considering that in higher plants each pollen grain is a haploid gametophyte of only two (max three) cells generated by the diploid sporophyte through a meiosis (microsporogenesis), pollen ephemeral life is limited at producing two gametes (microgametogenesis), possibly after being transferred to a plant different from the one that generated it (cross-pollination). Despite the smallness of its size and the shortness of its life pollen grains of any species are autonomous organisms, genetically different from any other and subject to the interaction with the environment. Pollen grains are exposed to natural selection and can influence the genetic constitution of the resulting sporophytic generation (Mulcahy 1979). The possibility to screen the individuals best adapted to specific environmental conditions is the concept which gametophytic selection is based on and proposed as alternative to the much wider used sporophytic selection for crop species (Hormaza and Herrero 1996). Although the studies on the effects of external factors on pollen biology are mainly focused on mature and/or germinated grains, environmental conditions affect pollen at all development stages, including the microsporogenesis (Pacini and Dolferus 2019).

Interaction of pollen with environmental factors is relevant not only for crop production on Earth but also for long term mission in Space. Indeed, pollen functionality has a key role in implementing bioregenerative life support systems (BLSSs) for long duration space-flight missions. Therefore, research on interaction between pollen and environmental factors, in addition to space factors as simulated microgravity (De Micco et al. 2006), is essential to realize the seed-to-seed cycle also on board spacecraft.

Considering recent projections of climate changes, a rising concern for gradual increases and abrupt variations of temperature is leading research to select genotypes tolerant to high temperatures or heat waves and to clarify the physiological processes involved in heat-stress responses (Hedhly et al. 2009; Mesihovic et al. 2016). Generally in plants, pollen functionality is strongly affected

by high temperatures, alone or in interaction with RH (Aronne 1999). More particularly, high temperatures are well known to affect pollen viability, pollen germinability and fertilization both in crops and in other plant species (Pérez et al. 2019; Aronne et al. 2015).

Pollen sterility induced by high temperature stress is an agricultural problem because it limits the productivity of many crop species (Pacini and Dolferus 2019). Negative effects of high temperature on pollen functionality constitute possible bottlenecks in plant life cycle of many species and conditions (De Micco et al. 2014; Aronne 2017). Therefore, a deep understanding on how high temperatures affect pollen functionality is essential to ensure food security.

At present, a special need to deepen pollen reaction to altered temperature conditions is relevant not only to predict the effect of gradual global warming on crop productions, but also to prevent the effect of sudden changes of temperatures during single producing cycles. Such events of heat waves are not rare in open field and in greenhouses and might strongly affect quantity and quality of crop production (Ledesma and Kawabata 2016).

Previous studies on the effect of high temperature at flowering, generally focus their attention on the functionality of the male gametophytes (Aronne et al. 2006; Djanaguiraman et al. 2019; Aronne et al. 2021) and are performed incubating mature pollen grains samples after their release from the anthers (Dafni 1992). However, negative effects of high temperature before pollen development have been described on microsporogenesis of several species (Porch and Jahn 2001; Szalay et al. 2019; Masoomi-Aladizgeh et al. 2020). Among crops, a considerable amount of studies has been conducted to assess heat stress on tomato pollen, because of its agronomic relevance and its exposure to temperature fluctuations during flowering, both in open field and in greenhouse (Firon et al. 2006; Paupière et al. 2017a). In tomato heat stress is also reported to interfere with tapetum formation, exine deposition and vacuolization of mature microspores (Giorno et al. 2013) and also with starch accumulation (Pressman et al. 2002). These heat stress-induced defects are produced during the microsporogenesis, may strongly affect the progression of male gametogenesis, and therefore the correct formation and

functionality of mature pollen. Notwithstanding the general knowledge on the effect of temperature on plant reproduction, specific studies aimed to investigate on possible relations between a heat stress occurred during the microsporogenesis and a later effect on the gametogenesis have been generally neglected.

Tomato is frequently used as model species for studies on interaction between high temperature and reproductive biology (Paupière et al. 2017b; Pham et al. 2020). In this work we grew dwarf tomato plants under finely controlled temperature conditions to deepen the timing of pollen ontogenesis and to test the hypothesis that high temperature occurring during microsporogenesis can affect pollen formation and especially subsequent functionality of the male gametophyte.

### 2.3. Material and methods

The experiment was performed in two equal growth chambers (VELP®, FOC 200IL). We used plants of *Solanum lycopersicum* 'Micro-Tom', a dwarf tomato selected to perform experiments in space. It is considered as an ideal model plant for experiments in confined environment because of its small habitus and its short life cycle (Matsukura et al. 2008). Among tomato cultivars, Micro-tom is used to elucidate the molecular mechanisms of reproductive biology traits in tomato because in addition to the small size and the short life cycle, it can be easily crossed with other tomato cultivars and genetically transformed (Sun et al., 2006). Moreover, the whole genome of Micro-Tom has been sequenced and made available in the 'TOMATOMICS' database (Kobayashi et al., 2014).

Plants were grown in plastic pots on a 1:1 (v/v) soil and perlite medium. A photosynthetic photon flux (PPF) of 200 µmol $m^{-2}$ $s^{-1}$ with a 16 h $d^{-1}$ photoperiod was provided by white LEDs and relative humidity was kept at 70% ± 5% throughout the whole experiment. At first, we grew 18 plants of Micro-Tom in a single growth chamber with an air temperature of 22°C ± 0.5°C (control temperature) generally considered as optimal for tomato (Sato et al. 2001; Matsuda et al. 2014). As soon as the primordia of the first inflorescences became visually distinguishable, we moved half plants in the other growth chamber in which all parameters were the same but the air temperature of 30°C ± 0.5°C (high temperature). This approach

was adopted to avoid possible effects of the two temperatures on vegetative phase of the plant growth and to ensure that the plants were exposed at the two different temperatures only during microsporogenesis. The high temperature treatment was designed and set in the growth chamber according to the screening for pollen tolerance in tomato reported by Paupière et al. (2017a) in which maximum average day temperatures for heat-tolerant tomato genotypes originated from location with hot environmental conditions varies from 28°C to 31°C.

We evaluated pollen functionality on a total amount of 72 flowers for each temperature treatment. For every single flower, the pollen from each anther was used for a single temperature treatment as specified below. To track pollen abortion, pollen viability and germinability during microsporogenesis and gametogenesis, therefore throughout flower lifespan, we collected flowers at four different flowering stages: a) Closed calix, b) Closed corolla, c) Anthesis, d) Post-anthesis (Fig. 1).

Pollen abortion at the end of microsporogenesis was measured using lactophenol-cotton blue stain that detects the presence of cytoplasm in pollen grains (Rodriguez-Riano and Dafni 2000). Pollen abortion was tested on 18 flowers for each temperature treatment occurring during microsporogenesis. Therefore, the pollen samples were collected from 36 anthers of different flowers buds with closed corolla. At this stage, we were able to compare the effect of the two temperature treatments occurring during microsporogenesis on pollen abortion, after microsporogenesis had ended. Each pollen sample was obtained by gently opening the undehisced anther with tweezers and spreading the pollen from a single anther directly on a 1 µL droplet of water previously placed on a slide. One droplet of 1µL of Lactophenol-cotton blue stain was added on each pollen sample and slides were mounted with a cover slip. We scored as non-aborted the pollen grains stained dark/blue and as aborted the ones that remained faint/colourless. Abortion percentage was measured counting at least 200 pollen grains per slide for a total of 36 slides (18 flowers x 2 temperature treatments). Scoring was made using an Olympus® BX-60 light microscope.

To compare the effect of the two temperatures during microsporogenesis on gametophyte functionality, we evaluated pollen viability and germinability after 72 hours of incubation at 22°C and 30°C. Half of the flowers developed under 22°C were incubated at 22°C and the other at 30°C. Similarly, half of the flowers developed under 30°C were incubated at 22°C and the other at 30°C. Overall, for each temperature treatment we incubated 27 flowers at 22°C and 27 flowers at 30°C. More specifically, incubated flowers were distinct into three categories corresponding to the stages of 1) closed corolla, 2 anthesis, 3) post anthesis. Each flower of the same phenological stage was excised from a single inflorescence, placed in open Petri dishes and incubated in two different growth chambers set at 22°C and 30°C with 70% RH.

To minimize possible inter-flower variations, 4 anthers per flower/bud were each used to assess: 1) pollen viability after 72 hours incubation at 22°C, 2) pollen viability after 72 hours incubation at 30°C, 3) pollen germinability after 72 hours incubation at 22°C, 4) pollen germinability after 72 hours incubation at 30°C.

Both for pollen viability and in vitro germination, three pollen samples of at least 200 grains were analysed per anther and scored with an Olympus® BX-60 light microscope.

To test pollen viability, we used diaminobenzidine (DAB) reaction because of its ease of use and its reliability to distinguish between viable and unviable pollen (Rodriguez-riano and Dafni 2000). For each sample, pollen from a single anther was spread on a slide. 1 µL droplet of DAB solution was added on each pollen sample and slides were gently warmed on a heating plate. When pollen was dry, slides were mounted with a cover slip and immediately microscope analysed. We scored as viable only pollen grains that turned totally black or dark brown.

In vitro pollen germination was tested through the hanging drop method using a germination medium modified from Karapanos et al. (2010) and Song et al. (1999) and optimized for Micro-Tom pollen. One droplet of germination medium was deposited on a cover slip and pollen from a single anther was released inside the droplet before turning the cover slip upside down. To prevent dehydration, slides with the hanging drops were stored in petri dishes sealed with parafilm. To evaluate

in vitro pollen germination, hanging drops were incubated at either 22°C or 30°C for 24 hours. Grains were scored as germinated when the pollen tube was longer than the pollen diameter.

To analyse pollen ontogenesis, we collected flower buds at different developmental stages, extracted the anthers with the help of an Olympus® SZX10 stereomicroscope and squashed them on microscope slides. We used different staining techniques on different anthers of single flowers. More specifically, to distinguish between well developed and aborted pollen we used aceto-carmine, toluidine blue and aniline blue staining procedures (Dafni 1992). Stained samples were observed with an Olympus® BX-60 light and epi-fluorescent microscope and results were cross-linked to get a comprehensive overview of the processes.

Data were analysed with IBM® SPSS Statistics. The Shapiro Wilk's test and Levene's test were respectively used to assess the normality and homogeneity of the variances of the datasets. Differences between the two temperatures during microsporogenesis on pollen abortion were compared with a t-test ($p < 0.05$). As concern pollen viability and germination, results were obtained from a three-factorial design (2 temperatures during microsporogenesis, 2 of incubation, 3 flower/bud types, 9 replicates) and significant differences were tested with a three-way ANOVA ($p < 0.05$). The Tukey HSD test ($p < 0.05$) was used for post-hoc analysis. Results expressed as percentage were converted with arcsine function.

## 2.4. Results

The Micro-tom plants grown at 22°C during the vegetative phase and subsequently grown at 22°C and 30°C during the reproductive phase, compared to those always kept at 22°, did not show visual differences in terms of growth rate, flowering time and flower morphology (colour and size).

Microscopic analysis showed that the microsporogenesis occurred in buds at the stage of closed calix and is completed at the stage of closed corolla both in flowers developed at 22°C and in those developed at 30°C.

The lactophenol-cotton blue test performed on pollen coming from flower buds with closed corolla, revealed that the number of not aborted/well-formed pollen grains was high in flowers developed under both temperature treatments. However, at closed corolla stage, percentage of not aborted pollen resulted only slightly lower ($p < 0,05$) at 30°C (97.64%) compared to 22°C (99.22%). Nevertheless, higher temperature during microsporogenesis did not lowered so much the number of well-formed pollen grains. Overall, the lactophenol-cotton blue test proved that higher temperature during microsporogenesis did not interfere so much with the first stages of pollen development and that the great majority of the grains in the anthers at the stage of closed corolla was well formed and functional in both temperature treatments.

Prolonging the temperature treatments on flowers up to the stage of anthesis and testing pollen viability with the DAB enzymatic reaction, we verified that most of the grains at anther dehiscence resulted viable in flowers from both temperature treatments. Percentage of viable grains in flowers developed at 22°C was statistically not different to that of flowers at 30°C (98.40% and 95.45% respectively, $p < 0.01$) (Fig. 2). Therefore, high temperature during flower development and microsporogenesis did not lowered pollen viability at the beginning of the anthesis.

The scenario changed completely when we collected the pollen from plants treated at the two temperatures during flowering/microsporogenesis development and subsequently incubated the grains at the same and cross changed two temperatures. Results of the DAB viability tests were analysed according to a three-factorial design including 2 temperatures during microsporogenesis, 2 incubation temperatures and 3 flower/bud types. The three-ways ANOVA revealed that the temperature during microsporogenesis resulted the main factor affecting pollen viability ($F = 6455.24$, $p < 0.05$) along all the flowering stages: closed corolla, anthesis, post-anthesis (Fig. 2).

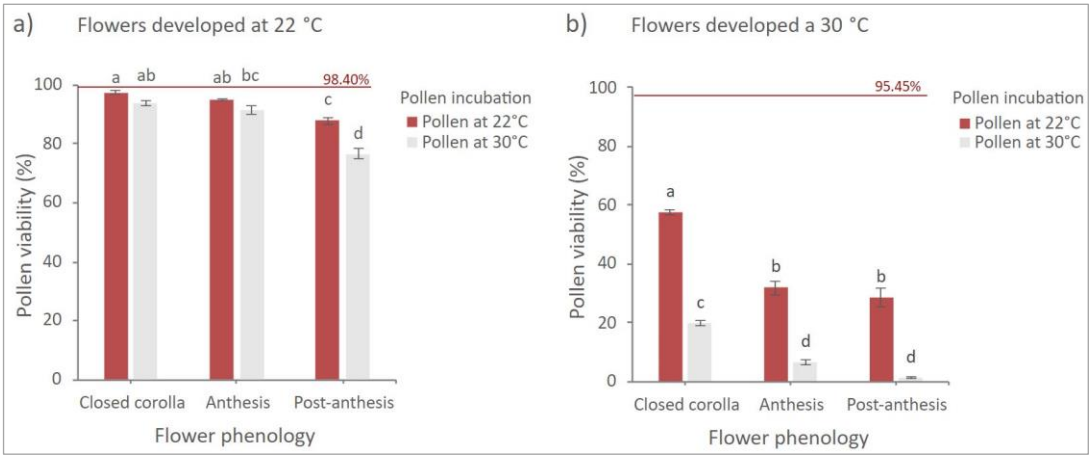


**Fig. 2.** Viability of pollen from flower at different phenological stages and incubated for 72 h at 22°C and 30°C. Data from flowers in which microsporogenesis occurred at 22°C (a) and 30°C (b). Horizontal lines show well developed pollen percentage at anthesis before incubation. Significant differences between phenological stages are expressed with different letters ($p < 0.05$). Bars represent ± SE.

When microsporogenesis occurred at optimal temperature (22°C), pollen viability gradually decreased remaining quite high along the whole flowering, ranging from 97.60% (closed corolla) to 76.77% (post-anthesis) (Fig. 2a). Conversely, higher temperature (30°C) during microsporogenesis (flower development) drastically reduced pollen viability from 57.49% (closed corolla) to 1.32% (post-anthesis) (Fig. 2b). According to the ANOVA, the main effect of the incubation temperature was also significant ($p < 0.01$) but it was strongly influenced by the interaction with the microsporogenesis temperature. Particularly, data showed that pollen in which microsporogenesis had occurred at optimal temperature, resulted more thermotolerant to the following exposure to incubation temperature; indeed, almost all pollen grains that lactophenol-cotton blue test detected as not aborted at closed corolla stage (99.22%) remained viable till anthesis stage without significant difference between 30° incubation (91.54%) at 22°C (95.10%) (Fig. 2a).

Higher temperature during microsporogenesis strongly reduced pollen thermotolerance to higher incubation temperature: indeed, pollen viability after 30°C incubation resulted significantly lower than 22°C in each phenological stage and

most importantly, almost all grain did not survived 30°C incubation at anthesis stage (6,56%) (Fig. 2b).

Overall, results showed that higher temperature occurring during microsporogenesis amplified the effect of the incubation temperature on pollen viability.

Effects on gametophyte functionality (pollen tube development) of high temperature treatment exerted during the microsporogenesis resulted significant. Data on pollen grains developed under the two temperatures and subsequently incubated to germinate at the same and at cross changed temperatures showed clear significant differences. More specifically, the three-way ANOVA revealed as significant ($p < 0,01$) the main effect of each factor and their interaction on germination percentage. According to the results, when microsporogenesis occurred at 30°C, almost no grain developed a pollen tube independently from incubation temperature and flowering stage (Fig. 3b).

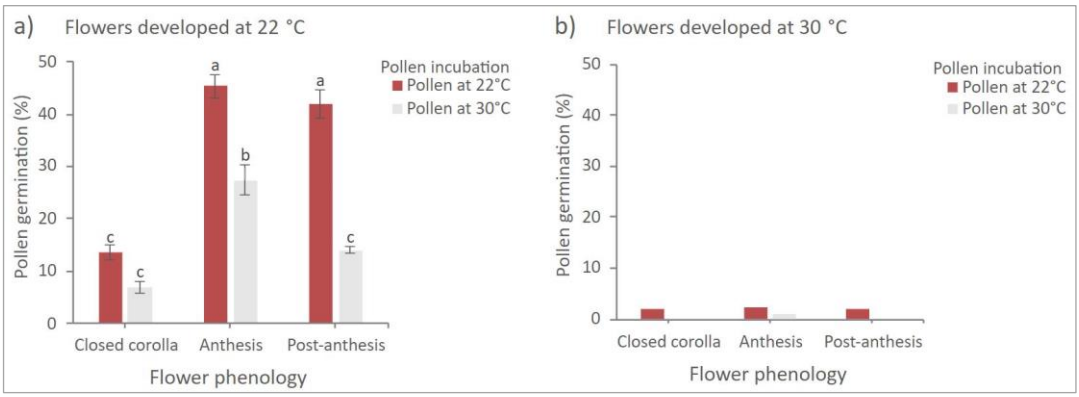


**Fig. 3.** Germinability of pollen from flower at different phenological stages and incubated for 72 h at 22°C and 30°C. Data from flowers in which microsporogenesis occurred at 22°C (a) and 30°C (b). Significant differences between phenological stages are expressed with different letters ($p < 0.05$). Bars represent ± SE.

When microsporogenesis occurred at optimal temperature, highest percentages of germinability occurred using pollen from flowers at the anthesis and percentages significantly decreased in pollen from flowers in post-anthesis (Fig. 3). Post-hoc analysis on the interaction between flower phenological stages and incubation temperature showed that at 22°C the decrease in pollen germinability was not

significant passing from anthesis to post-anthesis. Incubation at 30°C temperature not only significantly decreased pollen germinability in comparison to lower temperature but halved the percentage from anthesis (27.44%) to post-anthesis stage (13.96%) (Fig. 3a).

Data on germinability of pollen from flowers at closed corolla stage deserve special considerations. Percent germination of pollen from closed-corolla flowers resulted much lower than that from flowers at the anthesis and germinability apparently increased passing from the closed corolla to the anthesis stage. Such a phenomenon is not linked to the loss of viability (that instead resulted very high using the LCB and DAB tests). In-depth microscopy observations showed that when flowers are developed at 22°C, pollen in buds at the stage of closed corolla represent gametophytes not yet mature to develop the pollen tube (Fig. 1).

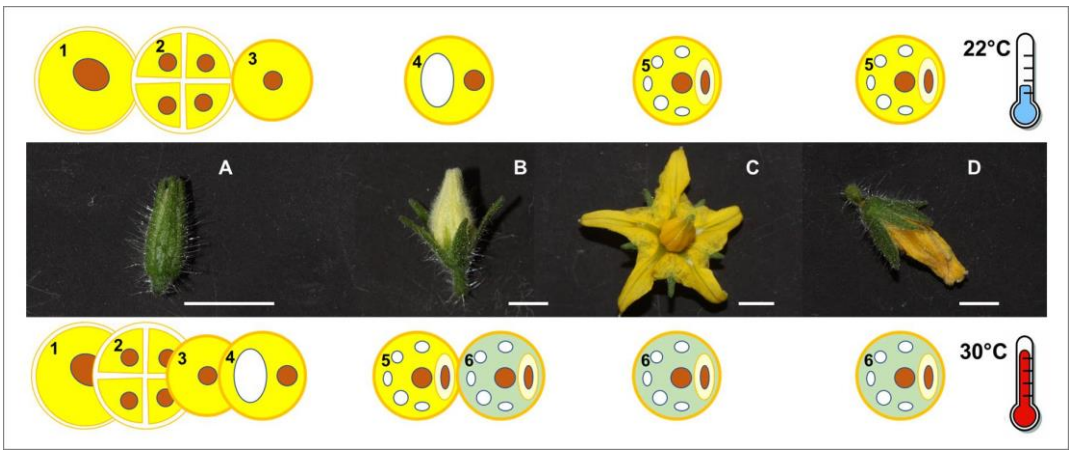


**Fig. 1**. Flowering stages and relative pollen development phases in flowers of tomato plants grown at 22°C and 30°C. Flowering stages: A) Closed calix, B) Closed corolla, C) Anthesis, D) Post-anthesis. Pollen developmental phases: 1) Pollen mother-cell, 2) Tetrad, 3) Uni-cellular microspore, 4) Vacuolated stage, 5) Bi-cellular pollen grain, 6) Dead pollen grain. Bars= 2mm.

Overall, results of germination tests revealed that both microsporogenesis temperature and the following exposure to incubation temperature are essential to ensure pollen tube development at anthesis stage when pollination should naturally occur. Further, microscope observations aimed to deepen the effects of high temperature on pollen ontogenesis and to identify possible cytological differences

during pollen development, revealed large differences on pollen development associated to temperature exposure of the anthers during microsporogenesis. Analysis of anther sections stained with aniline blue showed that high temperature conditions during microsporogenesis altered the timing of the developmental stages of pollen throughout flowering (Fig. 1). At the stage of closed calix, pollen mother cells, tetrads and uni-cellular microspores were observed in both treatments. Moreover, at the same stage, some microspores passed to the “vacuolated phase” only in anthers exposed to high temperature. At optimal temperature, pollen development resulted slower, and vacuolated microspores were observed from closed corolla stage on. Besides, temperature exposure of microspores affected the timing of the bi-cellular stage.

After the microsporogenesis, almost all microspores exposed to high temperature prematurely underwent the first haploid mitosis turning into mature pollen grains (bi-cellular gametophytes) at the stage of closed corolla. In flowers developed at 22°C, the bi-cellular stage became visible only from the anthesis stage, when mature pollen is expected to be ready for dispersal. From anthesis stage on, aniline blue stain highlighted differences in viability between the temperature treatments. In flowers developed at 22°C mature pollen was well-developed with densely stained cytoplasm from anthesis to post-anthesis stage. Differently, in anthers developed under high temperature, pollen cytoplasm drastically degenerated from closed corolla stage on and almost all pollen resulted aborted at post-anthesis.

Overall, cytological analysis allowed to clarify that the high temperature speeded up microspore transition into the male gametophyte (bi-cellular stage) and reduced pollen lifespan throughout flowering. In flowers developed at optimal temperature the presence of mature pollen in the anthers occurred as expected at the anthesis stage, while in flowers developed under high temperature pollen was ready for dispersal and fertilization when the flower was still a bud. At high temperature most of grains had lost their viability from anther dehiscence on and grains resulted too old to germinate at the anthesis stage.

## 2.5. Discussion

The experimental setup and design of using reversible combination of optimal and high temperature conditions during microsporogenesis and microgametogenesis allowed to disclose that a heat stress occurring only during microspore formation may become manifest later on the male gametophyte. Data showed that the drastic reduction of the male gametophyte functionality occurred even if the number of well-developed pollen grains at anther dehiscence resulted comparable to that of pollen developed at optimal temperature. Further microscopic analyses on pollen ontogenesis explained the relations between high temperature during microsporogenesis and high reduction of the male gametophyte functionality. The investigation revealed that heat conditions accelerate the senescence processes up to the point that pollen although well-developed, is mostly dead already at anthesis, so even before pollination dispersal. These results are critical for the achievement of the reproductive success in terms of seed/fruit production, especially considering that frequency of extreme weather events is expected to increase in a climate change scenario (Hedhly 2011). In such scenarios, summer crops such as tomato, will be more often exposed to heat waves even during the earliest stages of flowering when pollen is still developing. To date, other studies have tested sensitivity of pollen to thermal insults which frequently occurs for summer crops growing both in open field and in greenhouses (e.g. Porch and Jahn 2001; Mesihovic et al. 2016; Paupière et al. 2017a).

Reproductive success and the achievement of the seed-to-seed cycle is fundamental not only for crop cultivation also to reach the ambitious objective of realising complete self-sustaining cultivation systems for Space habitats (De Micco et al. 2014). Studies on plant biology in space reported that microgravity significantly affects pollen functionality interfering on several processes, including callose deposition at the end of the microsporogenesis (Kuang et al. 1995) or callose plugs formation in the male gametophyte (De Micco et al. 2006). Nevertheless, in addition to microgravity, interactions with other environmental factors have been identified as major contributors to male sterility in experiments conducted in space (Levinskikh et al. 2000; Campbell et al. 2001; Veselova et al. 2003). Sudden changes of

temperatures can occur on board of space stations during the period needed to close the seed-to-seed cycle. Considering that tomato is one of the crop species selected for cultivations in space, our study furnishes new insights to define the scientific requirements for future experiments on tomato reproduction in space and to correctly interpret the results.

Focusing on the way tomato plants respond to the environmental temperature during reproduction, previous research reports high variability in pollen response within 22-30°C temperature depending on how/when the temperature treatment was performed. For instance, Paupière et al. (2017a) reported significant loss of pollen viability on tomato when plants were exposed to high temperatures during the whole flowering. Conversely, Pham et al. (2020) did not found difference in terms of viability when pollen previously developed at optimal temperature was incubated at high temperature. Our results suggest that this variability in pollen thermo-tolerance is strictly linked to the pollen developmental stage targeted by heat treatment. Other studies on the effect of heat stress on tomato pollen report specific cytological anomalies including interferences during starch accumulation (Pressman et al. 2002) or exine formation and microspore vacuolization (Giorno et al. 2013); these processes occur during pollen ontogenesis and therefore might be involved in the lifespan and in the senescence speed of the pollen.

In the present work, pollen from flowers heat-treated during microsporogenesis always resulted significantly lower in viability compared to optimal temperature treatment while pollen in which microsporogenesis occurred at optimal temperature well tolerated both 22°C and 30°C incubation. Therefore, data showed that microsporogenesis occurring in the earliest stage of flower bud development represent a highly sensitive phase to high temperature exposure along the pollen ontogenesis. Although heat stress has already been reported to cause several dysfunctions in important monocot and dicot crops during early micropore stage, (Porch and Jahn 2001; Giorno et al. 2013; Jagadish et al. 2014; Szalay et al. 2019; Masoomi-Aladizgeh et al. 2020) none of these studies have described implications of these heat-stress induced defects on subsequent mature pollen viability and germinability, both essential to ensure fertilization success and crop productivity.

According to our results, it seems that uni-cellular microspores are less adapted to deal with environmental factors until their transition into bi- or tri-cellular pollen grains.

Different strategies in response to heat stress have been described in pollen to preserve viability from anthesis to stigma landing including dehydration, accumulation of osmolytes and synthesis of protective molecules such as heat stress proteins enforcing membrane stability (Pacini and Dolferus 2019). Differences between uni-cellular microspores and two or three-cellular male gametophytes in tolerating high temperatures, could be related to their different capability to express their haploid genes to overcome environmental constraints. Therefore, results of the present study should be taken into account to perform successfully gametophytic selection in breeding protocols for new cultivars best adaptable to heat stress both for future space missions and for cultivations on earth.

Our data showed that the effects of high temperatures were even more severe on pollen germinability than on viability. Such a phenomenon has been reported for species other than tomato (Aronne et al. 2015; Jiang et al. 2015; Djanaguiraman et al. 2018). However, in our case a few Celsius degrees over optimal temperature occurring in the earliest stage of flowering resulted fatal for the gametophytes therefore causing a reproductive failure. Considering that pollen grains detected as viable through DAB reaction do not necessarily develop pollen tubes (Dafni 1992), it is therefore reasonable that pollen from the high temperature treatment showing a very low viability did not germinate. Germinability of pollen developed at optimal temperature increased along flowering and reached the maximum percentage at anthesis. These results are in line with our cytological analysis in which we assessed that the highest germination percentage corresponds to the bi-cellular stage that in the optimal temperature treatment occurred at anthesis. Indeed, tomato pollen is generally dispersed as bi-cellular gametophytes and the second mitotic division only occurs after germination on the stigma. In the high temperature treatment, all the developmental stages resulted speeded up compared to optimal temperature treatment. In particular, the transition into the bicellular stage representing the condition of mature pollen had already occurred before anthesis. We thus highlight

that high temperatures during microsporogenesis also causes a premature transition of microspores into bi-cellular pollen to ensure mature pollen formation ready for dispersal but reducing pollen lifespan throughout flowering. Therefore, both direct effects of high temperature on the earliest stages of flower bud development and reduction of pollen lifespan throughout flowering can be responsible for a drastic loss in pollen viability and germinability.

Finally, the dwarf variety of tomato used for our experiment proved to be effective and further usable as a model for more studies on pollen functionality allowing to grow plants in small chambers where environmental parameters can be finely modulated and different temperature treatments can be performed during specific pollen developmental stages.

In conclusion, we successfully used tomato 'Micro-Tom' as model plant to contribute to the studies on the effects of heat stress on reproduction and verified the hypothesis that high temperature occurring during microsporogenesis can affect the subsequent functionality of the male gametophyte. Results also allowed to disclose that short periods of high temperature (as during the occurrence of heat waves) can accelerate pollen senescence processes priming a fatal shortening of the gametophyte lifespan and consequently a drop-off to zero of the viability before pollen is transferred by pollination.

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# Chapter 3

agronomy

Open Access Article

## High Temperature and Humidity Affect Pollen Viability and Longevity in *Olea europaea* L.

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# High Temperature and Humidity Affect Pollen Viability and Longevity in Olea europaea L.

## 3.1. Abstract

*Olea europaea* L. is a crop typical of the Mediterranean area having an important role in economy, society, and culture of this region. Climate change is expected to have significant impact on this crop which is typically adapted to certain pedo-climatic characteristics of restricted geographic areas. In this scenario, the aim of this study was to evaluate time-course response of pollen viability to different combinations of temperature and humidity. The study was performed comparing flowering time and pollen functionality of *O. europaea* from twelve cultivars growing at the same site belonging to the Campania olive collection in Italy. Pollen was incubated at 12 °C, 22 °C and 36 °C, in combination with 50% RH or 100% RH treatments for 5 days. Results highlighted that a drastic loss of pollen viability occurs when pollen is subjected to a combination of high humidity and high temperature, whereas 50% RH had less impact on pollen thermotolerance because most cultivars preserved a high pollen viability over time. In the ongoing climate change scenario, it is critical to assess the effect of increasing temperatures on sensitive reproductive traits such as pollen viability to predict possible reduction of crop yield. Moreover, results highlighted that the effect of temperature increase on pollen thermotolerance should be evaluated in combination with other environmental factors such as humidity conditions. The screening of the olive cultivars based on pollen thermotolerance resulted to be critical in the ongoing climate change scenario especially considering that the economic value of this species relies on successful fertilization and embryo development, and also that production cycle of *Olea europaea* can be longer than a hundred years.

**Keywords:** climate change; germplasm; olive; pollen viability; pollen functionality.

## 3.2. Introduction

Climate change will severely impact the Mediterranean Basin with an expected rise in temperatures in the range of 2–5 °C [1–3]. Besides a substantial warming, it has been estimated that climate change will result in a significant decrease in precipitation in this region [3,4], which might cause serious economic and ecological changes, influencing plant growth, the attack of pest and weed, and ultimately crop yield [5].

The olive (*Olea europaea* L.) is one of the most characteristic crops of the Mediterranean Basin, having a remarkable economic, social, and cultural impact. This species is widely spread and well adapted to the environmental conditions of the Mediterranean Basin. However, the predicted increase in ambient temperature due to global warming may affect plant physiology, phenology and reproductive biology of this crop, ultimately reducing its yield [6,7].

The Mediterranean region is characterized by a changeable climate, especially in spring season when daily temperatures can vary considerably. Moreover, it has been shown that inter-annual climate variations can affect flowering time and pollen production [8]. Indeed, previous studies have shown that the flowering time of *O. europaea* is highly dependent on the yearly spring temperatures, which are rising steadily over time due to global warming [9,10]. In this scenario, the olive phenology may provide useful indications to evaluate the influence of climate change on plant growth for the whole Mediterranean region, since the geographical limits of this cultivation approximately delimit the extent of the Mediterranean climate in Eurasia and North Africa [11,12]. Since the different olive varieties are adapted to specific climatic, edaphic, and lithological conditions, the possible variations occurring in a climate change scenario would have a significant impact on the distribution of these varieties and, consequently, on their growth and productivity [13,14]. This especially expected for some old varieties cultivated in narrow geographic niches with specific micro-climatic characteristics [15]. Indeed, it has been shown that these varieties exhibit greater vulnerability to both short-term climate variability and long-term climate change [16].

The olive has significant phenotypic and genetic variability [17,18]. Factors including plant longevity, limited selection pressure, and limited replacement with new genotypes, have reduced genetic erosion and favored the preservation of genetic diversity of olive varieties [19]. Italy has the richest olive collection including about 700 different varieties. Among the Italian regions, Campania has one of the largest collections [20,21] and many of these varieties are characterized by an extensive morphological diversity and adaptation to local environmental conditions [22]. Olive plants can produce an abundant number of flowers but generally only a small percent (1–2%) of them set normal fruits that reach maturity [23,24]. The success of a flower to become a fruit mainly depends on the pollination and fertilization processes. Previous studies reported that pollen germination and pollen tube growth are sensitive to elevated temperatures [25,26]. Moreover, it has been shown that the combination of relative humidity (RH) and temperature can affect pollen viability of other species [27–29]. Temperature also influences both drupe development and oil composition in olive. For example, in very hot sites, olives can show early pigmentation because of the rapid degradation of chlorophyll due to high temperatures [30], whereas, in sites with lower temperatures, olive oil has a high content of unsaturated fatty acids [31]. Temperature can also influence the aromatic components of olive oil, reducing the content of volatile substances [32]. Hence, it is arguable that the growing environment is crucial in expressing the typical characteristics and quality of olive cultivars [33]. In most crop species, including *Olea europaea* L., the production of fruits and seeds rely on pollen functionality. Since pollen viability and germinability are both essential to ensure fertilization, the interaction of pollen with extreme weather events can significantly limit crops productivity in the current climate change scenario [34]. Among extreme weather events, heat waves during early stages of pollen development, can reduce pollen functionality in *Solanum lycopersicum* L. resulting in a drastic loss of pollen germinability [35].

Most of the studies evaluating the effect of environmental factors on olive pollen mainly focused on temperature [26,36], whereas very few studies evaluated the combined effect of temperature and humidity [37]. Specifically, Koubouris et al. [37]

studied the effect of pre-incubation temperature and humidity on olive pollen before *in vitro* germination. However, authors did not test the combined effect of high temperature and high humidity, which could severely affect pollen functionality. Among protocols to assess pollen functionality, the use of diaminobenzidine (DAB) reaction is an efficient method to assess pollen viability responses towards environmental factors [38,39]. Because of its ease of use, DAB method can be useful for large screening of pollen viability, such as the case of studies with numerous cultivars. In the current climate change scenario, the aim of this study was to highlight possible differences among different olive cultivars in the time-course response of pollen viability to different combination of temperature and humidity treatments. We used pollen from 12 olive cultivars belonging to germplasm of Campania region in Southern Italy and growing at the same site. We hypothesized that both temperature and humidity would affect pollen viability with possible changes due to treatment duration and cultivars. Moreover, we hypothesized that high humidity would enhance the negative effect of high temperature on pollen viability and longevity.

## 3.3. Material and Methods

### *3.3.1 Plant material and flowering monitoring*

The experiment was performed using pollen from 12 olive cultivars of Campania region. Plants belong to the open-field collection conserved at "Improsta" Regional Experimental Farm, in Eboli (SA) (40 ° 33 '29 "N; 14 ° 58' 28" E at 15 m.a.m.s.l.). We selected olive cultivars representative of 4 provinces of Campania region and covering the bioclimatic diversity of the whole area (Table 1).

**Table 1**. Cultivars of *Olea europaea* L. from 4 provinces of Campania region in Italy.

| N | Province | Name |
|---|---|---|
| 1 | Avellino | Marinese |
| 2 | | Ravece |
| 3 | | Ogliarola |
| 4 | Benevento | Ortice |
| 5 | | Ortolana |
| 6 | | Racioppella |
| 7 | | Femminella |
| 8 | Caserta | Caiazzana |
| 9 | Salerno | Biancolilla |
| 10 | | Carpellese |
| 11 | | Pisciottana |
| 12 | | Salella |

For each cultivar, the duration of flowering was determined by a procedure reported by Rapoport e Rallo 1991 [40] with some modifications: the flowering phenology of three different branches with south exposure and approximatively 100 flowers per branch was observed. We considered as starting day of flowering when 10% of flowers per each branch were open, and as last day when 100% of flowers per branch were open. The duration of flowering (number of days) was then averaged based on measurements on the three different branches.

In the field, data of minimum, maximum and medium temperature (°C) and RH (%) during May 2021 were recorded from the agro-meteorological regional station of Eboli (SA), located at “Improsta” Regional Experimental Farm.

The collection of pollen samples was carried out from 18 to 28 May 2021. Pollen was shed from the inflorescences in Petri dishes through pollen vibrators. Sampling

was performed in the morning and Petri dishes were transported to the laboratory in few hours. For each cultivar, pollen was collected from branches with north, south, east, and west exposure from three different plants.

*3.3.2 Temperature and humidity treatments*

Pollen samples were incubated under six different combinations of temperature and humidity for a total of 5 days. According to the temperature measured during the flowering season at the experimental farm, three temperature treatments were tested: 12 °C, 24 °C and 36 °C. More specifically, 12 °C and 24 °C were tested to simulate the averaged minimum and maximum temperatures of May, whereas 36 °C was chosen to simulate a possible scenario of global warming and heat waves. In combination with temperature treatments, the pollen from the 12 cultivars was incubated at 50% RH and 100% RH to simulate the effect of dry-sunny and wet-rainy days occurring during the flowering season.

Temperature treatments were performed using three separated incubators (VELP, FOC 200 IL) set with 12°C, 24°C, 36°C respectively. In each incubator, to achieve 50% or 100% RH conditions we enclosed the bulk samples of pollen in two separated plastic containers containing: a) a beaker with Mg $(NO_3)_2$ saturated solution to reproduce 50% RH, b) wet tissues to achieve 100% RH.

*3.3.3 Analysis of pollen viability*

Pollen thermotolerance and longevity of the 12 olive cultivars were assessed performing viability tests at 1-, 3-, and 5-days incubation. Pollen viability was assessed through diaminobenzidine (DAB) reaction [38,39]. Each pollen sample was gently collected with a brush from the petri dish and placed onto 10 µL droplet of water on a microscope slide. One droplet of 10 µL of DAB reagent was then added on each sample. Successively, the microscope slides were gently warmed on a heating plate (set at 50°C) and mounted with a cover slip. The viability of pollen at sampling ($T_0$) was assessed to compare possible differences in initial pollen functionality among cultivars and to have a reference point for comparing the effect of temperature, RH, and their interaction throughout the incubation period. We

scored as viable the pollen grains stained black/brown and as not viable the ones that remained faint/colourless. The percentage of pollen viability was measured at different incubation time, counting at least 100 pollen grains per microscope slide on a total amount of 6 slides per cultivar per treatment.

*3.3.4 Data analysis*

Data were analysed using Excel ver. 16 (Microsoft Corp., Redmond, USA) and SPSS Statistics ver. 21 (IBM Corp., Chicago, USA). Percentage data of pollen viability was preliminary converted with arcsine function. The Shapiro Wilk's and Levene's tests were used to assess the normality and homogeneity of variance, respectively. The influence of the different categorical independent variables (i.e., cultivar, time, temperature, and humidity), and their possible interactions on pollen viability was analyzed using the ANOVA. Pairwise comparisons were performed with Tukey's HSD test ($P > 0.05$) to identify differences among treatments and cultivars.

## 3.4. Material and Methods

*3.4.1. Climatic parameters*

Temperature and humidity data recorded in May 2021 at the experimental farm are shown in Figure 1. The average values of the daily minimum, medium, and maximum temperature were 11.8 °C, 17.7 °C, and 23.3 °C, respectively. The highest temperature was recorded on May 24, reaching a peak value of 31.8 °C, while lowest temperature was 7.5 °C and was recorded on May 21. On average, the daily minimum, medium, and maximum RH values were 50.6%, 73.1%, and 91.29%, respectively. Overall, the daily RH values were comparable between the different days, except for a considerable decrease of daily RH values that was recorded from 20 May to 25 May (Figure 1).

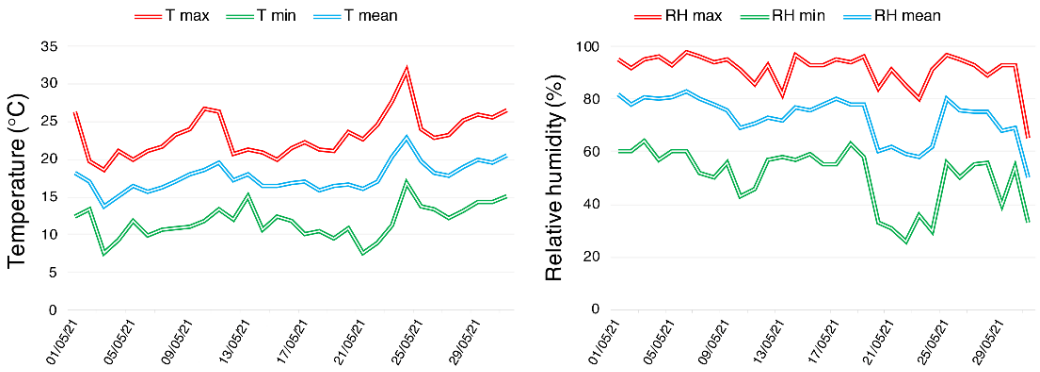


**Figure 1.** Daily trend of maximum (red line), minimum (green line) and mean (blue line) temperature and humidity measured in May 2021 at "Improsta" Regional Experimental Farm, in Eboli (SA) (40 ° 33 '29 "N; 14 ° 58' 28" E, at 15 m.a.s.l.).

### *3.4.2. Flowering time*

Figure 2 shows the duration of flowering time of the different olive cultivars considered in this study. On average, the flowering time among cultivars was 7 days. Moreover, the shortest and the longest duration of flowering were recorded in 'Pisciottana' (3 days) and 'Femminiella' (10 days), respectively (Figure 2).

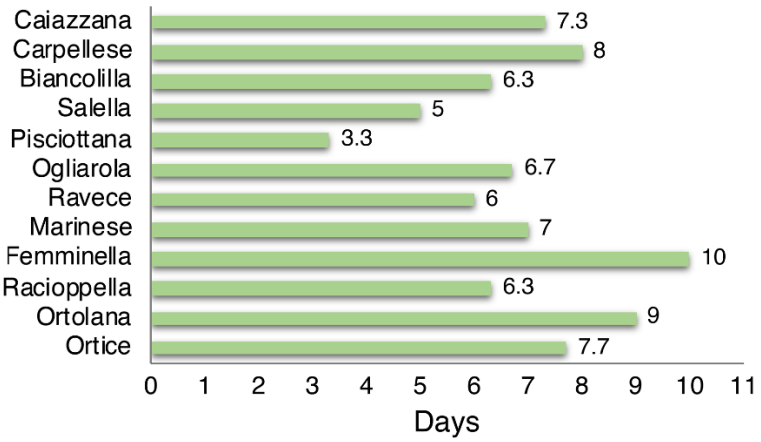


***Figure 2.*** *Duration of flowering in 12 olive cultivars from Campania region in Italy.*

*3.4.3. Pollen viability*

According to the ANOVA, all factors tested in this study (i.e., cultivar, temperature, and humidity) had a significant effect on pollen viability over time (Table 2). The viability tests performed on pollen at $T_0$ showed significant differences between the 12 cultivars (Figure 3). However, despite these differences, pollen viability of all cultivars at $T_0$ ranged between 84% and 95%, except for 'Marinese' in which pollen viability was 64%.

**Table 2.** Analysis of variance for the effects of cultivar, relative humidity (RH), temperature, or their interaction on pollen viability of *Olea europaea*.

| Factor | Sum of Squares | Sig. |
|---|---|---|
| Cultivar | 165808.8 | *** |
| Time | 548079.9 | *** |
| RH | 157134.8 | *** |
| Temperature | 28848.1 | *** |
| Temperature * RH | 27059.9 | *** |
| RH * Cultivar | 8670.5 | NS |
| Temperature * Cultivar | 13972.0 | NS |
| Temperature * RH * Cultivar | 14478.7 | NS |

NS, or *** indicate nonsignificant or significant at $P < 0.001$, respectively.

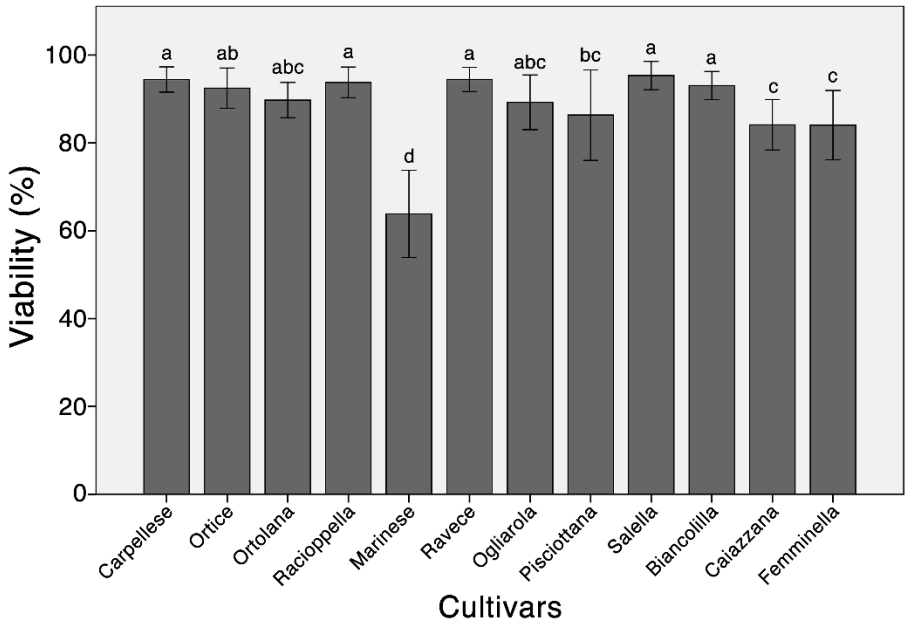


**Figure 3.** Pollen viability of 12 cultivars of *Olea europaea* L. at sampling time ($T_0$). Letters indicate significant differences between cultivars ($P < 0.05$). Each data represents the mean ± SE (n = 6)

For each cultivar, time-course response of pollen viability was affected by the different combinations of temperature and RH over 5 days incubation. As concern treatments with 50% RH, pollen viability showed no significant decrease over time compared to pollen at $T_0$ in all cultivars. Notably, pollen preserved a high viability (~80%) both at high (24 – 36 °C) and low (12 °C) incubation temperature over time (Figure 4). A lower pollen viability was found only in 'Marinese' but it was comparable to pollen viability at $T_0$.

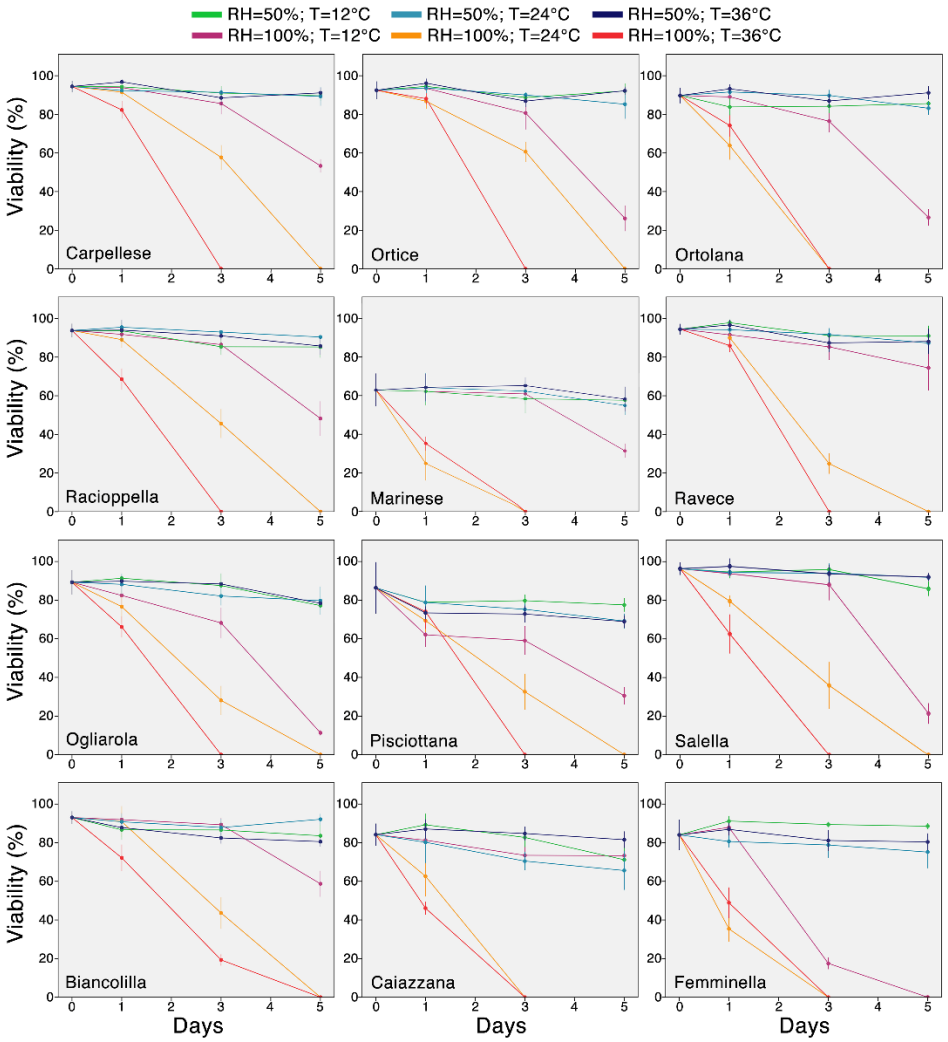


**Figure 4.** Viability of pollen from 12 olive cultivars incubated at six different combinations of temperature and humidity for 5 days ($T_1$-$T_5$) from sampling ($T_0$). Each line shows the mean ± SE (n = 6).

Differently from treatments with 50% RH, pollen subjected to 100% RH showed a significant decrease over time in all cultivars with differences due to the incubation temperature. Overall, results showed a drastic loss of viability when pollen was subjected to a combined effect of high humidity (100%) and high temperature (36 °C). Indeed, pollen grains incubated at 100% RH and 36 °C completely lost their viability after 3-days incubation in almost all cultivars. Pollen viability was preserved

for more than 3-days incubation at 100% RH and 36 °C only in ‘Biancollilla’, although with low values (~20%) (Figure 4).

A drastic loss of pollen viability also occurred when pollen was subjected to 100% RH and 24 °C, showing considerable variability over time depending on the cultivar. Compared to pollen at 100% RH and 36 °C, pollen at 24 °C showed higher viability over time and preserved its viability longer than under 36 °C. Indeed, pollen under 100% RH and 24 °C remained viable up to 5-days incubation in most of the cultivars except for ‘Marinese’, ‘Caiazzana’ and ‘Ortolana’, in which pollen grains become unviable at 3-days incubation (Figure 4).

Differently from 24 °C and 36 °C, the combination of 100% RH and 12 °C showed a more gradual loss of pollen viability over time in most of the cultivars. More specifically, pollen viability remained high over 3-days incubation and never decreased below 60%, except for ‘Femminella’. Interestingly, pollen incubated at 100% RH and 12° C showed no significative difference compared to treatments with 50% RH up to 3-days incubation (Figure 4).

## 3.5. Discussion

### *3.5.1. Climatic parameters and flowering*

Recent studies indicate that nearly all European regions will be troubled by the impact of climate change [27,41,42]. In this scenario, the Campania region in Italy has already experienced an increase in minimum temperatures of approximately 1.4 °C from 2005 to 2017 [43]. This situation, in agreement with research showing a dramatic global warming since the 1980s [13,44], poses concerns regarding the impact that climate change can have also on restricted geographical areas such as that of the Campania region.

*O. europaea* is a typical Mediterranean species whose economic production cycle is extremely long, and fruit production relies on pollen efficiency and fertilization success. Therefore, studies on the interaction between flower biology and environmental parameters involved in climate change scenario are relevant.

Our results on flowering period (time and duration) are comparable with those reported for several olive cultivars of the Campania region in the year from 2009 to 2010 [45], who showed that the average duration of flowering was 7 days and occurred during the second half of May. It is known that environmental factors can affect many aspects of inflorescence development, pollination, and fertilization; in particular, high temperatures can influence the timing of phenological phases such as leaf formation and flowering in many species including *O. europaea* [46–48]. Our data showed that in the last decade the flowering time and duration of the olive cultivars considered in this study did not change. However, studies on other species highlighted that short periods of high temperature do not affect flowering phenology but can reduce pollen lifespan so drastically that grains are already dead at the time of anther dehiscence [35]. Indeed, it has been shown that the formation of pollen tetrads and bi-nucleate olive pollen is very sensitive to small increments in temperature in May, when the heat demand for flowering is nearly fulfilled [49]. In addition, besides the satisfaction of heat requirements, it is possible that different olive cultivars require the fulfilment of other conditions for starting the flowering process, such as a mean temperature above 15 °C during the week before the anthesis [12,49]. It should be also considered that the suitable temperature range for metabolic process in *O. europaea* is rather narrow with an optimal temperature interval of approximatively 10 °C (from 20 °C to 30 °C) [50]. Therefore, when new cultivars are introduced in specific areas, it is critical to consider climatic requirements and flowering time of these cultivars.

### *3.5.2. Climatic parameters and flowering*

The response of pollen to environmental factors is critical in *O. europaea*, considering that self-incompatibility represent a common phenomenon in most of the olive cultivars [51]. Indeed, olive pollen from different cultivars needs to survive along its journey from the stamen to the stigma of different flowers to ensure the formation of seeds and drupes. Considering the great genetic diversity of olive cultivars in Campania region, pollen viability represents a crucial feature for the selection of cultivars to be used as pollen donors in a climate change scenario.

Our results showed that the decrease of pollen viability over time is highly dependent on the exposure to different combinations of temperature and RH. Pollen viability at sampling time exceeded 80% in almost all cultivars tested in this study. Interestingly, pollen viability of these cultivars was higher compared to most commercial cultivars from Europe which generally range between 60% and 70% [52–54]. The low genetic erosion due to the limited replacement of typical olive cultivars with new genotypes in Campania region, may have conserved relevant reproductive traits such as pollen viability. On the other hand, commercial cultivars have been mostly selected to overcome self-incompatibility issues to increase plant productivity [55], but probably overlooking reproductive traits such as pollen viability during breeding programmes.

Our results are in agreement with previous studies, showing that high temperatures can reduce pollen viability more than low temperatures [29,54]. It has already been reported that temperatures above 22 °C can reduce by 50% the initial pollen viability of *O. europaea* in 1-3 days [54]. However, previous studies mostly overlooked the effect of humidity, since RH was not explicated or fixed, and its effect could not be evaluated. Conversely, our study focused on the effect of different combinations of temperature and humidity to disentangle their effects on pollen functionality. Overall, with this approach we found that RH has a substantial influence on pollen thermotolerance over time.

Previous studies have shown that the exposure of olive pollen to high RH during pre-incubation can significantly decrease pollen germinability *in vitro* at low temperatures [37]. Differently, our results showed that high humidity severely increase pollen sensitivity to both low and high temperatures. Specifically, the combination of high temperature (36 °C) and high RH (100%), significantly reduced pollen viability already after 1-day exposure and pollen was completely unviable after 3 days in most of the cultivars. Conversely, pollen exposed to low RH (50%) preserved high viability (~80%) both at higher (36-24 °C) and lower (12 °C) incubation temperatures over 5 days. These differences in pollen longevity could be

related to specific mechanisms adopted by pollen to survive hostile environmental conditions [56]. Indeed, pollen grains under low humidity environments can enter a state of complete or partial arrest of metabolic processes associated with a high resistance to environmental stresses [56]. This phenomenon might explain the significant differences of pollen thermotolerance to high or low humidity.

Water content of pollen grains generally decrease after flower anthesis and anthers dehiscence when pollen is exposed to the environment [57]. Therefore, from anthesis on, the possibility of pollen to enter in a quiescent state of development and resist to unfavourable temperatures depends on pollen exposure to environmental humidity. Indeed, especially in self-pollinating species, pollen is dispersed in a well-hydrated state and remains metabolically active; this pollen generally is more sensitive to environmental stresses and has reduced viability since it needs to germinate rapidly upon landing on the stigma of the same flower [58–60] Differently, in cross-pollinated species such as *O. europaea*, pollen needs to survive for a relative long time and, therefore, it needs to reduce its water content to maintain a metabolically inactive state during its journey to the stigma of other flowers. In this case, the balance between the content of water, osmolyte compounds, and stabilizing proteins in pollen grains make the cellular content “glassy” and all the metabolic activities result slowed down [61]. However, this “glassy” state, responsible for increasing pollen longevity, is influenced by both humidity and temperature exposure of pollen grains [62–63]. Indeed, when pollen is exposed to low temperatures, this state of glassy cytoplasm can be achieved also in conditions of high humidity, and this would explain why pollen exposed to 12 °C and 100% RH preserved a high viability up to 5 days in most of the cultivars we tested. As regard temperatures of 24 °C or 36 °C, their negative effects on pollen viability becomes evident over time only when in combination with exposure to high RH.

Overall, the differences in thermotolerance found between the different olive cultivars can be linked to the capability of pollen in adopting specific strategies to face heat stress including dehydration, accumulation of osmolytes, and synthesis of

protective molecules such as heat-shock proteins (HSPs). Numerous studies highlighted the key role of HSPs in activating heat-stress responses in reproductive cells of several plant species [64–68]. To date, HSPs have been identified in *O. europaea* but only in vegetative tissues [69]. In the present study, it is likely that the different responses of pollen to temperature and humidity found between cultivars are due to differences in heat-stress response pathways. In this regard, the screening of HSPs gene expression and synthesis of olive cultivars would provide a better understanding of the molecular mechanisms adopted by pollen to cope with heat stress. Moreover, such insights could be useful to select suitable olive cultivars as pollen donor to be used in a climate change scenario.

### 3.6. Conclusion

Despite the increase in temperature in Campania region over the last decade, no significant change in flowering time of *O. europaea* was found compared to previous studies. However, a drastic loss of pollen viability was found under high temperature and humidity conditions. Overall, the decreasing trend of pollen viability under the different combinations of temperature and humidity was comparable between cultivars, except for few cases. Specifically, most of the olive cultivars showed a significant decrease of pollen viability already after 24h incubation under 36 °C and 100% RH, and a complete loss of viability after 3-days incubation in the same conditions. Interestingly, pollen exposed to low RH (50%) preserved high viability both at high and low incubation temperatures over 5 days, indicating that pollen thermotolerance is affected by humidity conditions. In a current scenario of climate change, it is critical to evaluate the effect of temperature on reproductive traits to predict the future impact of global warming on crop yield; on the basis of the results obtained we could therefore state that the cultivars that showed greater tolerance to extreme temperatures and humidity was Biancolilla (RH 100% -T ° 36 ° C), while ‘Carpellese’, ‘Ortice’, ‘Racioppella’, ‘Ravece’, ‘Ogliarola’, ‘Pisciottana’ and ‘Salella’ also showed good tolerance, in conditions of RH 100% and T ° 24 °C. However, it becomes evident that other environmental factors such as humidity must be considered when evaluating pollen thermotolerance. Moreover, considering the key

role of the heat-shock proteins in heat-stress responses, further studies must investigate the molecular mechanism adopted by olive pollen to cope with environmental stresses.

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# Chapter 4

## FLOWERING AND POLLEN RESILIENCE TO HIGH TEMPERATURE OF APRICOT CULTIVARS

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# Flowering and pollen resilience to high temperature of apricot cultivars

## 4.1. Abstract

*Prunus armeniaca* L. is widely cultivated in the Mediterranean area including Southern Italy where local cultivars are recognized for their excellent quality. Gradual warming and abrupt variations of seasonal temperatures are expected to significantly impact the Mediterranean area with potential implications on many crops including apricot. In this scenario, the identification of physiological processes involved in heat-stress responses and the selection of genotypes resilient/tolerant to high temperatures and heat waves are necessary. The aim of this study was to investigate possible differences in flowering phenology and the effect of different temperatures on pollen functionality of 13 apricot cultivars traditionally cultivated in the Campania region (Southern Italy). According to phenological data collected in the field, the studied cultivars were grouped in early, intermediate, and late flowering cultivars. Single flower anthesis was on average 4.9 days in early and intermediate flowering cultivars, whereas late flowering cultivars showed a shorter duration (4.0 days). Pollen of flowers at balloon stage showed to have a viability uniformly high among all cultivars. To investigate on possible effects of temperature during the effective pollination period (EPP), pollen from the different cultivars was pre-incubated at 5 °C, 15 °C, and 25 °C for 48-h and germinability was then assessed after further 24-h *in-vitro* germination at the same temperature treatments. The first two temperature values correspond respectively to the minimum and maximum average temperatures of the local area; while 25 °C simulated the heat waves recently recorded in the farm during apricot flowering periods. As regard pollen germinability, 15°C revealed to be the most suitable temperature for apricot pollen to germinate within the EPP. Conversely, 5 °C and 25 °C significantly reduced pollen germination in most cultivars and particularly in intermediate flowering cultivars. Noticeably, a few cultivars showed no difference in pollen germination under the different temperature treatments, preserving high pollen germinability (>70%) even

at high temperatures. Overall, our findings highlighted that pollen germination is extremely sensitive to temperature with significant variations among apricot genotypes. Overall, results confirmed that reproductive traits such as pollen germinability represent an important parameter to consider for monitoring fruit production, in the processes of cultivar selection for new orchard plantations and in breeding projects. Moreover, traditional apricot cultivars as those of the Campania region confirmed to be a precious source of genetic diversity potentially possessing a significant pollen resilience to temperature changes.

## 4.2. Introduction

European apricot cultivation has experienced a significant cultivar turnover in the last decades mainly focusing on the cultivars that best meet the new market requirements such as early and late ripening (Ruiz et al., 2018). This phenomenon is common to other species, has intensified crop production but has also caused a drastic reduction in agrobiodiversity that, in turn, increased vulnerabilities of agricultural systems to climate variation (Pingali, 2012).

The Campania region (Southern Italy) is one of the main apricot-growing areas in Italy and among the most important in Europe. In this area apricots have been cultivated since ancient times leading to the selection of a multitude of traditional cultivars that are still cultivated by local farmers and widely recognized for their excellent qualitative and quantitative performances also outside the local area (Di Vaio et al., 2019).
The most representative cultivars of the Campania region are collected in the germplasm repository of apricot trees at Eboli (Southern Italy). This collection is of great economic and scientific interest for fruit breeders and researchers focused on genetic diversity and resilience to climatic changes of apricot cultivars in the whole Mediterranean area (Rao et al 2010; Di Vaio et al 2019)

The projections of climate changes in the Mediterranean areas (e.g., Giorgi and Lionello, 2008) are rising concerns not only for gradual warming but also for abrupt

variations of seasonal temperatures. Consequently, researchers commit to select genotypes tolerant to high temperatures or heat waves and to clarify the physiological processes involved in heat-stress responses (Hedhly et al, 2009; Mesihovic et al. 2016).

Climate change is impacting on the biological dynamics of plants showing phenological aberrancies (Walther et al., 2002; White et al., 2009). In pome and stone-fruit trees of temperate regions, extreme weather events are reported to affect reproduction in key phases (as the erratic break of the bud dormancy) that ultimately results in plant yield reductions and financial losses (Byrne et al., 2000; Legave et al., 2013; Bartolini et al.,2019). Temperature exposure over time is the main driver for spring development, including bloom timing and leaf-out (Rathcke and Lacey 1985, Bertin 2008). This makes the species with spring flowering phenology the most sensitive and responsive to climate change in temperate regions (e.g., Schwartz et al. 2006).

Among reproductive traits, pollen interaction with the environment represents a potential bottleneck in plant life cycle that can limit the reproductive fitness and productivity in plant species (Aronne, 2017). Indeed, pollen functionality has been widely reported to be extremely sensitive to environmental constraints (Aronne et al., 2015; Asma, 2008; Egea et al., 1992; Pirlak, 2002). Particularly, pollen viability and germination are mostly dependent on temperature and humidity exposure of pollen grains (Aronne et al., 2021; Güçlü and Koyuncu, 2017; Sorkheh et al., 2018). As concern temperature, pollen thermo-tolerance is strictly related to the pollen developmental stage targeted by the heat treatment and consequently to the flowering stage in which temperature treatment is performed. Indeed, high temperatures have drastic implications on the functionality of mature pollen if the temperature treatment is performed in the earliest stage of pollen development (Iovane and Aronne, 2021).

Relative humidity of air, especially combined with temperature treatments, can play a key role in determining pollen sensitivity to environmental factor exposure (Aronne et al., 2006). Particularly, in some wind-pollinated species such as *Olea europaea* L. (in which pollen is dispersed in a low hydrated state), the exposure to high

humidity triggers pollen sensitivity to temperature and drastically reduces pollen longevity (Iovane et al., 2021; Pacini and Dolferus, 2019; Bassani et al., 1994).

In most crop species, including *P. armeniaca*, fruit and seed production rely on a successful pollen functionality (Ruiz and Egea, 2008). More specifically, pollen needs to stay viable and germinable along its journey from the anther to the stigma, succeed in all steps of gametogenesis and double fertilization to generate new seeds and consequently trigger fruit tissue formation. Indeed, both pollen viability and germination are strongly correlated with fruit and seed set (Herrera et al., 2018; Paupière et al., 2017). However, viable pollen grains might not be able to produce a pollen tube, and this is the reason why pollen viability and in vitro-germination should be both tested to evaluate pollen functionality.

Pollen viability and germination represent reproductive traits with a different ecological meaning and different response to the environment: viability tests are generally used to evaluate the effective fulfilment of the pollen grain development process; in vitro-germination tests measure the capability of pollen to elongate a pollen tube and potentiality to succeed in double fertilization (Dafni, 1992). Therefore, insights on both viability and germinability are relevant to have a complete overview of pollen responses toward different temperatures during the period from pollen dispersion till pollen germination onto the stigma. More specifically, pollen viability and germination should be assessed within the effective pollination period (EPP), defined as the number of days during which pollination is effective in fruit set because pollen functionality, stigmatic receptivity, and ovule longevity overlap (Sanzol and Herrero, 2001).

In the current climate change scenario, the aim of this study was to investigate on possible differences in flowering and pollen functionality of traditional apricot cultivars. More specifically, we hypothesized that temperatures treatments (both higher and lower than the average temperature of the flowering season) would affect pollen germination throughout the EPP of *P. armeniaca*.

## 4.3. Material and Methods

### *4.3.1. Plant material*

The experimental trial was conducted on different apricot genotypes belonging to the open field germplasm collection at "Improsta" Regional Experimental Farm, in Eboli (Southern Italy) (40° 33' 29" N; 14° 58' 28" E, 15 m a.s.l.). The research activities in the field were carried out in 2021, starting on February 15$^{th}$ and ending on March 30$^{th}$. The study was performed on 13 apricot cultivars, namely: 'Don Aniello', 'Ottavianese', 'Ceccona', 'Sona Campana', 'Vitillo', 'Zi Francesco', 'Zi Luisa', 'Portici', 'Pellecchiella', 'Boccuccia Liscia', 'Portici II', 'Boccuccia Spinosa', 'Scialò'. Individual plants of all apricot cultivars were twelve-year-old, grafted on Myrabolan 29C (*Prunus cerasifera*) and vase-trained (4 m × 4 m spaced). For our study we used five single-tree replications per cultivar.

Data of minimum, maximum and medium temperature (°C) and rainfall (mm) were recorded throughout the experimental period at the agro-meteorological station located at the "Improsta" Regional Experimental Farm. Climatic data were compared with those recorded for the period from 2018 to 2020.

### 4.3.2. Flowering phenology

Considering that plants of all cultivars were subjected to the same local environmental factors, we monitored their phenology to assess possible differences in flowering related to the different genotypes. To avoid possible interference between flowering time and sun exposure, we standardized the monitoring of plant phenology by selecting only South-East facing branches.

For each cultivar the average length of the whole blooming period was determined by marking a branch with approximatively 100 flowers and recording as "start of blooming" the day with the first opening flower and as "end of blooming" the day with the last opening flower. According to blooming timing, the 13 cultivars were grouped into early, intermediate, and late flowering group.

In addition, to determine the duration of single flower anthesis, we chose and marked 8 flower buds on 8 different mixed branches for each plant. We then marked

and monitored every other day each flower from “pink bud” to “all petals fallen” stage (BBCH 57 - 69, Pérez-Pastor et al., 2004).

### *4.3.3. Pollen viability and germinability*

The effect of temperature on pollen functionality of the 13 apricot genotypes was assessed through in vitro pollen viability and germinability tests. Three temperature treatments were performed on apricot pollen: 5 °C, 15 °C and 25 °C. We chose 5 °C and 15 °C treatments because they represent respectively the minimum and maximum mean temperature recorded at the "Improsta" farm during apricot flowering of the years preceding that of this study. The third temperature treatment (25 °C) was chosen to investigate pollen response in a possible scenario of heatwaves that actually occurred in the same area during last years.

Pollen was collected from flowers sampled on the trees of the 13 apricot cultivars. More specifically twigs with flower buds were cut in the morning and transported to the laboratory in a few hours. Per each genotype, not less than 100 flower buds in “balloon” stage were collected (BBCH 59, Pérez-Pastor et al., 2004). At “balloon” stage, anther resulted light yellow, turgid and still undehisced, therefore preventing pollen dispersal and possible pollen damaging (including dehydration) during transportation. Upon arrival at the laboratory, anthers were detached from flower buds, were distributed in three petri dishes, and were left to dehisce by desiccating them in a closed container filled with dry silica gel. This approach allowed anthers to release pollen within few hours and to produce three bulk samples of pollen per cultivar in the same day as flower sampling from the trees.

Pollen viability was assessed through diaminobenzidine (DAB) reaction (Rodriguez-Riano and Dafni, 2000) at “balloon” stage to compare possible differences in initial pollen functionality among cultivars and to have a reference point for comparing the effect of the different temperature treatments on pollen germination over the EPP. We evaluated pollen viability on 12 pollen samples per cultivar. Each pollen sample was taken from a single bulk per cultivar and spread into a 10 µL droplet of water

previously placed on a slide. One droplet of 10 µL of DAB reagent was added on each pollen sample and slides were gently warmed on a heating plate and mounted (Dafni, 1992). We scored as viable the pollen grains stained dark brown and as not viable the ones that remained faint/colourless. Viability percentage was measured counting at least 100 pollen grains per slide.

The EPP for apricot flowers is reported to vary among cultivars from 2 to 8 days (Sanzol and Herrero, 2001) Considering that no data is available on the EPP of the apricot genotypes from the Campania region, we decided to analyse the effect of the temperature treatments on pollen tube formation considering 72 hours as plausible EPP. More in details, pollen from the selected cultivars was pre-incubated at 5 °C, 15 °C and 25 °C for 48 hours and germination was evaluated after further 24-hours *in-vitro* germination at the same temperature treatments, respectively. Pollen used for germination tests came from the same bulk samples in which viability was previously assessed. More specifically, pollen from bulk samples was placed on a series of Petri dishes lined with a solid medium made of 0.9% agar and 15% sucrose in water. For each cultivar, six Petri dishes were split in three different incubators (VELP®, FOC 200 IL) set at 5 °C, 15 °C, 25 °C, respectively, and 70% RH. Pollen grains were scored as germinated when pollen tubes were longer than the pollen diameter. For each cultivar, we scored at least 100 pollen grains per each of the four Petri dishes incubated at the same temperature.

*4.3.4. Statistical analysis*

Data were analysed with Microsoft Excel and IBM® SPSS Statistics. The Shapiro Wilk's was used to assess the normality of the datasets. Both for viability and germination, data resulted normally distributed within each of the three datasets (early, intermediate, and late flowering cultivars). In each dataset homogeneity of variance was verified by Levene's Test. Data expressed as percentage were preliminary converted with arcsine function. Differences in pollen viability among cultivars were compared with one-way ANOVA ($P < 0.05$). As concern germination, results were obtained in a two-factorial design (4 temperatures × 13 cultivars) and

significant differences were tested with a two-way ANOVA ($P < 0.05$). All post-hoc analyses were performed with Tukey HSD test ($P < 0.05$). Statistical analysis of data on flowers at anthesis was performed using one-way ANOVA ($P < 0.05$) and Duncan's multiple range test ($P < 0.05$).

## 4.4. Results

### *4.4.1. Flowering phenology*

Meteorological data recorded at the 'Improsta' farm during the apricot flowering period in 2021 showed that the average temperature was 10.5 °C, the average max temperature was 16.1 °C, the average min temperature was 4.8 °C, and 0.4 °C and 22.9 °C were the daily minimum and maximum temperature, respectively (Fig.1). During the flowering period, rainfalls were concentrated only in a few days and did not interfere with the data collection and pollen sampling.

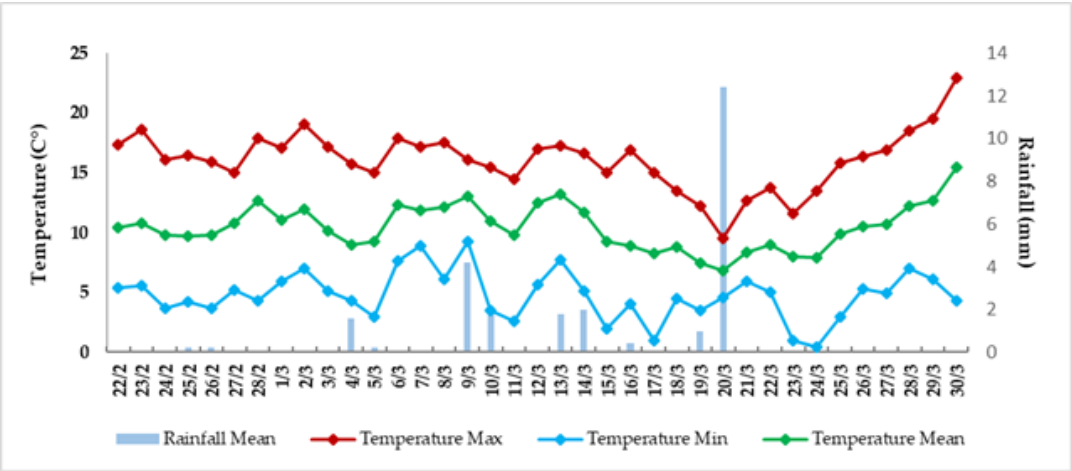


**Fig. 1.** The daily trend of the maximum, minimum, and mean temperature (C °) and total rainfall (mm) calculated in the period from February 22nd to March 30th 2021 at the experimental site of 'Improsta' farm.

According to phenological data, we grouped the studied cultivars into three categories of blooming time: a) early flowering cultivars ('Sona Campana', 'Don Aniello', 'Ottavianese and 'Ceccona') with bloom starting from February 22nd to 27th;

b) intermediate flowering cultivars ('Vitillo', 'Zi Francesco', 'Portici', 'Zi Luisa' and 'Pellecchiella') with bloom starting from March 2nd to 6th; c) late flowering cultivars ('Boccuccia Liscia', 'Boccuccia Spinosa', 'Portici II' and 'Scialò') with bloom starting on March 8th (Figure 2). 'Sona Campana' showed the longest blooming period (13 days) and was the first cultivar to bloom (February 22nd) whereas 'Vitillo' showed the shortest blooming period (9 days) (Fig. 2).

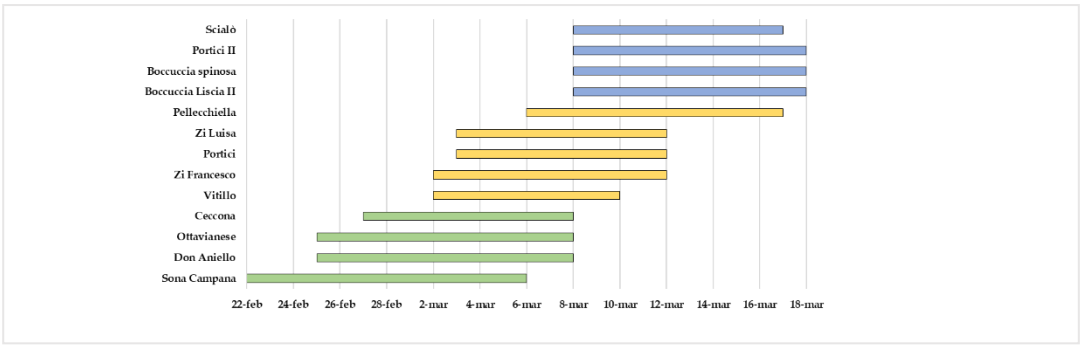


**Fig. 2**. Blooming period length of the studied apricot cultivars grouped as: early flowering (February 22nd to 27th) (green bars), intermediate flowering (March 2nd to 6th) (yellow bars), and late flowering (March 8th to 18th) (blue bars).

Overall, the length of single flower anthesis (from "pink bud" to "all petals fallen") was in average 4.61 days, whereas 'Zi Francesco' (5.6 days) and 'Scialò' (2.88 days) showed the longest and shortest duration, respectively (Fig. 3). Focusing separately on the three groups, early cultivars did not show any statistically significant difference in blooming period with an average duration of 4.94 days. The intermediate cultivars showed on average an anthesis duration similar to the early cultivars (4.9 days); however, two of them turned out to be respectively longer ('Zi Francesco' = 5.6 days) and shorter ('Zi Luisa' = 3.5 days) than the others. Late flowering cultivars showed on average a shorter anthesis compared to the other cultivars (3.97 days); among them the anthesis of 'Scialò' cultivar (2.88 days) lasted less than the others.

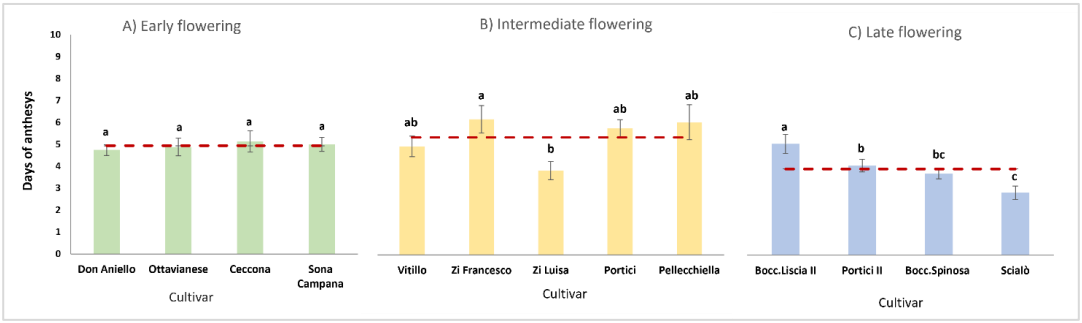


**Fig. 3.** Single flower anthesis in early (green bars), intermediate (yellow bars), and late flowering cultivars (blue bars). Dashed lines represent the average duration of the anthesis for each group. Data are expressed as mean number of days ± SE. Significant differences between cultivars within each flowering group are expressed with different letters ($P < 0.05$).

### *4.4.2. Pollen viability and germinability*

Viability of the pollen grains collected from flowers just before the anthesis was uniformly high in all the apricot cultivars ranging from a minimum of 82.18% ('Zi Luisa') to a maximum of 96.37% ('Sona Campana') (Fig. 4). Notwithstanding some statistically significant differences highlighted in each of the three groups, on average pollen viability at the anthesis remained hight throughout the apricot flowering period.

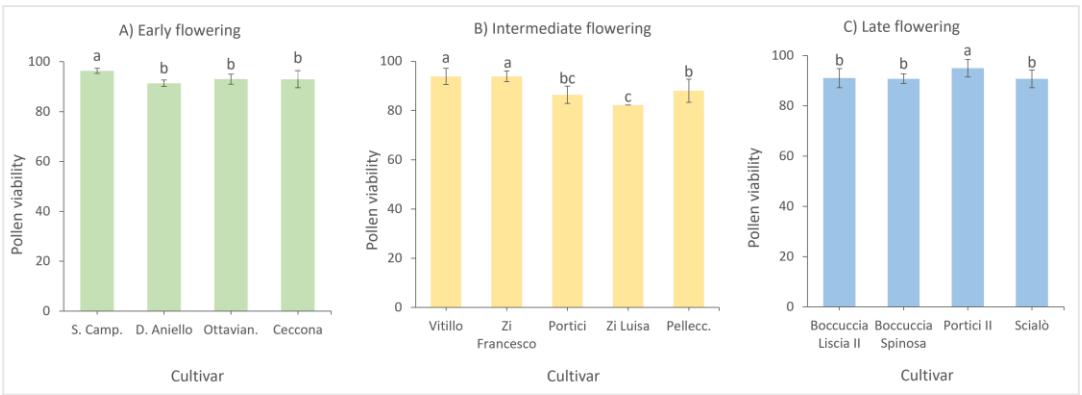


**Fig. 4.** Viability of pollen from flower sampled at "balloon" stage in early (A), intermediate (B), and late flowering cultivars (C). Significant differences between different cultivars within each flowering group are expressed with different letters ($P < 0.05$). Bars represent means ± SE.

According to these results, for all cultivars pollen was considered of good quality and suitable to analyse the effects of temperature on gametophyte development by means of germination tests. The response of pollen of *P. armeniaca* to 5 °C, 15 °C and 25 °C incubation within the simulated EPP (48 h pre-incubation and 24 h *in-vitro* germination) varied significantly among cultivars. Overall, pollen germination ranged from a minimum of 5.47% (‘Ceccona’) to a maximum of 83.50% (‘Portici II’) (Fig. 5). The temperature of 15 °C, reproducing the maximum mean temperature during flowering, revealed to be the most suitable temperature for apricot pollen to germinate within the EPP; indeed, the highest germination percentage was recorded at 15 °C (Portici II, 83.50%).

Compared to 15 °C, the temperatures of 5 °C and 25 °C (reproducing the minimum and the peak temperature during flowering, respectively) significantly reduced pollen germination in most cultivars (Fig. 5). Differences in pollen germination were particularly evident in the intermediate flowering cultivars in which overall germination was lower compared to cultivars of the other flowering groups with the lowest values occurring at 25 °C (Fig. 5B).

Among the 13 cultivars, only three (‘Ottavianese’, ‘Zi Francesco’ and ‘Portici II’) showed no difference in pollen germination to the different temperature treatments within the EPP. Moreover, two of them (‘Ottavianese’ and ‘Portici II’) resulted also to be the cultivars conserving high pollen germinability (> 70%) at high temperatures, therefore showing the best resilience to temperature changes during flowering.

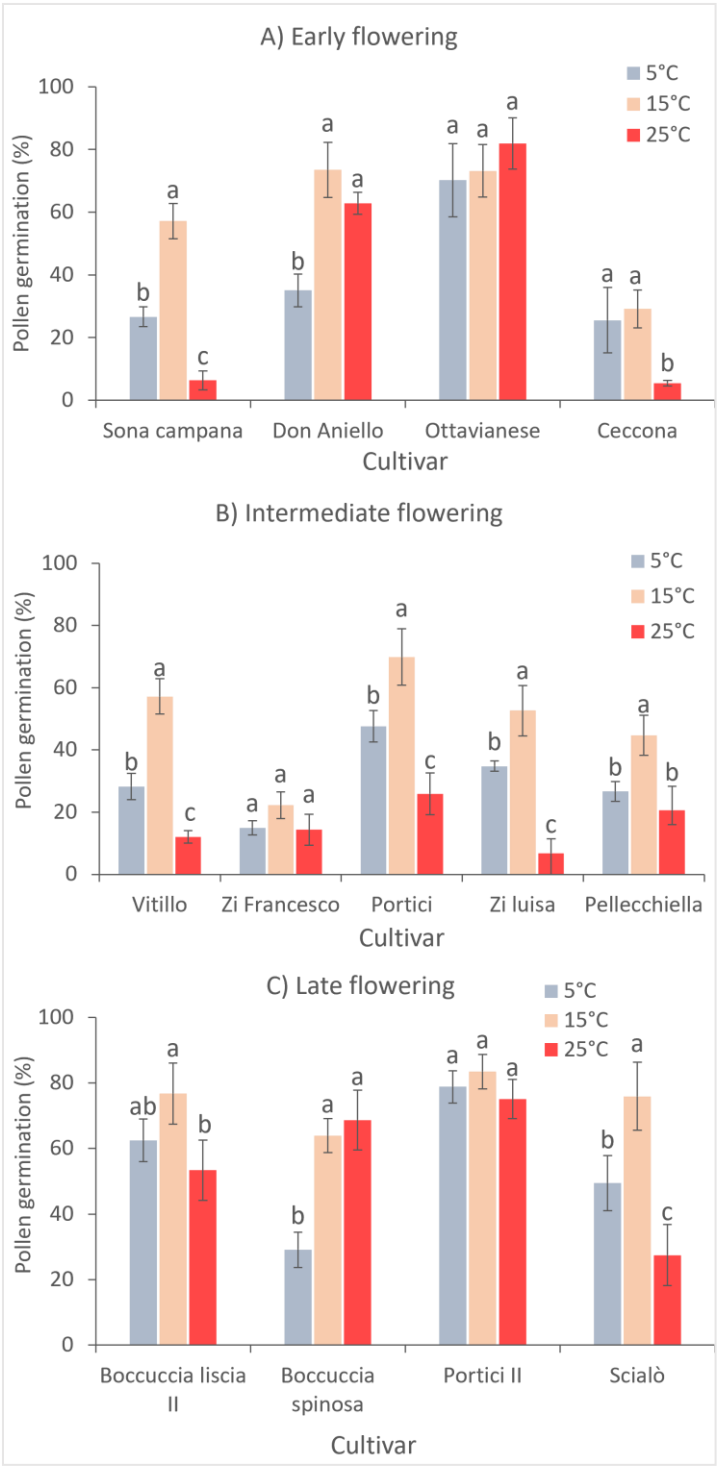


**Fig. 5** Germinability of pollen sampled from flowers at "balloon" stage in early (A), intermediate (B), and late (C) flowering cultivars. Significant differences within each cultivar are expressed with different letters ($P < 0.05$). Bars represent means ± SE.

## 4.5. Discussion

The possibility to analyse reproductive traits of different apricot genotypes by using trees of the same age and growing at the same place allowed us to reliably compare their flowering phenology and pollen functionality. Insights on the blooming time are valuable in the process of cultivar selection for breeding projects, new orchard plantations, or in predicting possible future scenario in established orchards. However, blooming time is a complex phenomenon related to environmental and endogenous cues such as development and physiological status of plants (Amasino and Michaels, 2010). Chill temperatures are known to be much involved in regulating the blooming time and inadequate chilling due to warm autumn temperatures results in delayed and erratic budbursts during the following spring (Cannell and Smith, 1986; Heide, 2003; Darbyshire et al., 2017). It is reported that one of the main effects of the warmer climate in orchards is the faster bud development and earlier blooming of fruit trees, including apricots (Szalay and Papp 2006). These phenomena rise the risk of frost damage, the consequences of which depend on several factors including cultivar genotypes. In our study, we compared and categorized all cultivars according to their blooming time. Overall, our data on seasonal period and duration of the flowering agreed with those previously reported on some of the same apricot cultivars (Pellegrino et al., 2004; Cirillo et al., 2010). Nevertheless, some contrasting results have been found in literature, as for the cultivar ‘Pellecchiella’ that was classified both as early flowering (Cirillo et al., 2010) and as late flowering (Pellegrino et al., 2004). In our study, the coexistence in the same place of so many cultivars represented by plants of the same age, allowed to homogenize the effects of the environmental factors on the flowering processes and to consider the differences among cultivars as imputable only to their different genotypes.

Weather conditions can affect floral morphology and fruit set in apricot with a significant variability among cultivars (Legave et al., 2013; Ruiz and Egea, 2008), and warm pre-blossom temperatures are reported to limit the pistil development of apricot flowers (Rodrigo and Herrero, 2002). No aberration in flower morphology

was visually observed in any of the analysed cultivars. However, significant year-by-year variations in apricot floral traits are plausible and previously reported for other cultivars (Ruiz and Egea 2008); therefore, it would be worth to repeat flowering analyses on all our cultivars for several subsequent years.

In breeding programmes new commercial cultivars have been mostly selected to overcome self-incompatibility issues and increase plant productivity (Marchese et al., 2016), whereas other reproductive traits, including pollen functionality and its interaction with environmental factors, have been generally overlooked.

Our data on pollen viability of flowers at dehiscence ('balloon stage') showed that both microsporogenesis and early microgametogenesis processes had successfully occurred in all tested cultivars. Similar studies on viability of pollen from flowers at the 'balloon stage' were performed in Turkey on foreign apricot varieties reporting much lower viability percentages compared to the traditional cultivars analysed in our study (Asma, 2008; Yaman and Turan, 2021). Such differences might be related to several reasons. One might be linked to the type of test used to evaluate pollen viability: we chose the diaminobenzidine (DAB) reaction to test pollen viability considering that the tetrazolium salts, although used for a long time as also in the mentioned studies, it is now being replaced by more reliable enzymatic reactions (Dafni 1992). Moreover, the lower pollen viability in foreign cultivars tested in Turkey compared to cultivars in our study, might be linked to the low genetic erosion due to the limited replacement of typical apricot genotypes with new commercial cultivars in Campania region. Finally, we cannot exclude that the differences in pollen viabilities might be related to the interactions between the cultivars and the local weather conditions during the years of the mentioned studies.

High levels of pollen viability in all cultivars allowed us to go further focusing on the effect of temperature on pollen functionality during a time compatible with the EPP of apricot species.

Results highlighted large differences among cultivars and, for most of them, 15 °C revealed to be the best temperature for pollen germination. Such results are in line with those of previous studies evaluating germination of apricot pollen at different temperatures (Pirlak, 2002). However, it is worth to consider that 15 °C corresponds to the maximum mean temperatures previously recorded at the 'Improsta' farm

during the apricot flowering periods. Consequently, it seems that pollen from traditional cultivars of Campania region is resilient to the mean high temperatures occurring recently in the field. Throughout the flowering period, no specific trend was found in pollen germination to the minimum mean temperatures previously recorded at the 'Improsta' farm during the apricot flowering periods. Best resilience was showed on average by cultivars belonging to the early and late flowering groups. Nevertheless, further investigations are required to deepen these results. Moreover, it must be considered that pollen can react differently to temperature during pre-incubation treatments or during *in-vitro* germination. Despite pollen germination decreased at 5 °C, it is already reported that low temperature can increase pollen longevity and preserve pollen capability to germinate even after long storage (Dutta et al., 2013; Khan et al., 2013; Sharafi, 2011). In this regard, additional experiments should test possible effects of low temperature before and after the activation of pollen tube formation. Finally, our cultivars showed to be significantly affected by high temperatures corresponding to the heatwaves locally recorded in the recent years during the apricot flowering periods. Overall, our findings rise concerns especially considering the current scenario of climate change. In addition, our results proved that different cultivars could have different reactions to heatwaves and that pollen from different genotypes can be more or less resilient to the increase in maximum mean temperatures.

In conclusion, pollen functionality of apricots is highly sensitive to environmental conditions showing that temperature can drastically reduce reproductive success in apricot cultivars. Our results also showed that in apricot, within the gametophytic generation, pollen germination is the phase most affected by temperature. Moreover, results further highlighted that the traditional genotypes of apricot (such as those of the Campania region) despite their lower productivity compared to commercial cultivars, must be considered as precious source of agrobiodiversity because of their best conserved reproductive traits. Among them, pollen reaction to temperature should no longer be neglected in the processes of cultivar selection for new orchard plantations or breeding projects.

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# Appendix 1A

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## A novel device to study altered gravity and light interactions in seedling tropisms

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# A novel device to study altered gravity and light interactions in seedling tropisms

**Abstract**

Long-duration space missions will need to rely on the use of plants in bio-regenerative life support systems (BLSSs) because these systems can produce fresh food and oxygen, reduce carbon dioxide levels, recycle metabolic waste, and purify water. In this scenario, the need for new experiments on the effects of altered gravity conditions on plant biological processes is increasing, and significant efforts should be devoted to new ideas aimed at increasing the scientific output and lowering the experimental costs. Here, we report the design of an easy-to-produce and inexpensive device conceived to analyze the effect of interaction between gravity and light on root tropisms. Each unit consisted of a polystyrene multi-slot rack with light-emitting diodes (LEDs), capable of holding Petri dishes and assembled with a particular filter-paper folding. The device was successfully used for the ROOTROPS (for root tropisms) experiment performed in the Large Diameter Centrifuge (LDC) and Random Positioning Machine (RPM) at ESA's European Space Research and Technology Centre (ESTEC). During the experiments, four light treatments and six gravity conditions were factorially combined to study their effects on root orientation of *Brassica oleracea* seedlings. Light treatments (red, blue, and white) and a dark condition were tested under four hypergravity levels (20*g*, 15*g*, 10*g*, 5*g*), a 1*g* control, and a simulated microgravity (RPM) condition. Results of validation tests showed that after 24 h, the assembled system remained unaltered, no slipping or displacement of seedlings occurred at any hypergravity treatment or on the RPM, and seedlings exhibited robust growth. Overall, the device was effective and reliable in achieving scientific goals, suggesting that it can be used for ground-based research on phototropism-gravitropism interactions. Moreover, the concepts developed can be further expanded for use in future spaceflight experiments with plants.

## 1 INTRODUCTION

In their natural environment, plants have evolved to adapt to a wide number of different physical environmental factors that interact with each other (Vandenbrink et al., 2014). While most of these factors (including, but certainly not limited to, temperature, light, and water availability) can vary widely, the acceleration of gravity that pulls the plants toward the center of the Earth is a constant with the value of 1*g* ≈ 9.81 m/s$^2$. Recently, plant biologists increased efforts to improve the knowledge of plant responses to altered gravity conditions, mainly microgravity (De Micco et al., 2014; Izzo et al., 2019; Medina et al., 2021; Vandenbrink & Kiss, 2016; Zabel et al., 2016). The impetus for increased studies in this field comes from the necessity of using plants for long-term manned missions in space, due to their crucial role in Bioregenerative Life Support Systems (e.g., De Micco et al., 2009, 2012; Paradiso et al., 2014).

Not only microgravity, but also hypergravity experiments, provided relevant insights into the gravity perception and response of plants, including the interaction of gravitropism with other tropisms (Fitzelle & Kiss, 2001; Hattori et al., 2020). Additionally, in certain situations, hypergravity can be used to extrapolate responses to microgravity, according to the gravity continuum principle as well as through the reduced gravity paradigm (van Loon, 2016). Notwithstanding the clear negative effect that extreme acceleration has on plants, experimental data showed that crucial processes such as pollen tube formation (Musgrave et al. 2009) explored up to 35*g*, and even seed germination and subsequent growth can still occur at up to 10,000*g* (Waldron & Brett, 1990). Another advantage of using hypergravity for studying gravity related phenomena is its greater accessibility compared to real microgravity while being able to address comparable scientific questions. On Earth, hypergravity is easily achievable in centrifuges, whereas microgravity can never be achieved for a long period of time because 1*g* force is omnipresent, thus preventing the achievement of a weightless environment.

Considering that access to space experiments is very limited, screening studies, pre-flight experiments, and hardware testing are generally performed in simulated

microgravity conditions (Böhmer & Schleiff, 2019). Random positioning machines (RPMs) and clinostats are the most approachable systems to simulate microgravity on Earth (Kiss et al., 2019). However, both systems cannot provide a gravity-free condition but are based on a continuous change of the gravity vector orientation. More specifically, simulated microgravity using RPMs relies on gravity vector averaging to zero by continuously reorienting the gravity vector's trajectory of the samples (Borst & van Loon, 2009).

In hypergravity conditions, the force due to gravity is enhanced while its vector orientation is not altered. Several facilities are available to study plants in hypergravity, each with their own limitations and advantages. Standard centrifuges allow simple experiments with enclosed seedlings, but suffer from vibrations, steep *g*-gradients, poor ventilation and cannot include lights (Nakabayashi et al., 2006; Tamaoki et al., 2014). In these conditions, tissue morphology and gene expression responses to hypergravity have been studied for short durations (24 h) (Matsumoto et al., 2010; Toyota et al., 2007). The limited volume of standard centrifuges usually does not allow plants to grow beyond their seedling stage, especially for species other than *Arabidopsis thaliana*. Moreover, plant growth to adult stage requires water, gas exchange, light, and sufficient space for root anchoring and leaf exposure to light, all of which cannot be easily provided in standard centrifuges.

Long-running experiments with several plants necessitates custom build centrifuges, such as the MIJ-17, or the Large Diameter Centrifuge (LDC) of the European Space Agency (ESA) (Frett et al., 2016; Takemura et al., 2017). Experiments in the ESA's LDC benefit from a semi-controlled environment, large gondolas, and the possibility to set several different hypergravity levels per run with a maximum of 20*g* (van Loon et al., 2008; van Loon & Dowson, 2019). A particular advantage of the LDC is the possibility of lighting and monitoring inside the gondolas, enabled by a power supply and both USB and ethernet connections. Large diameter centrifuges contribute to reduce the quantity of the artefacts of centrifugation, for example in terms of inertial shear forces (van Loon et al., 2003) and gradient of the net force experienced along the biological sample containers not placed in the center of the gondolas.

To conduct plant biology experiments in the LDC, hardware designed according to the requirements of the specific experiment needs to be developed for use in the gondolas. With both the size (50 × 50 × 72 cm) and weight (80 kg) restrictions being quite generous, the hardware to be implemented in the LDC can either be sturdy and heavy, or flexible and light. Design decisions depend on the specific experiment and need to balance criteria as measurement accuracy, resilience, safety, feasibility, and costs. Generally, hardware made with light material is easy to make and adjust but must be designed to prevent possible collapse under the weight of hypergravity.

Hardware design must also consider that plants need to be secured against the pull of hypergravity. The possibility of dislodged plants is especially a concern for seeds before germination and seedlings grown on vertical agar plates. To achieve a successful experiment, the design of the hardware to be implemented in the LDC gondolas must fulfill all scientific requirements. Such a process applies also to plant biology experiments and can require substantial time and funds from research teams investigating hypergravity effects on plants. Therefore, the whole scientific community involved in studying the effects of altered gravity on plants might benefit from sharing the concept, design details, and test results of a hardware used for a successful experiment.

In this article, we share the design of an easy-to-produce and inexpensive device equipped with an adjustable LED lighting system, designed for plant experiments in the LDC gondolas and the large-size RPM. This hardware was first and successfully used for the ROOTROPS (for root tropisms) experiment aimed at studying the combined effect of gravity and light quality on root tropisms of *Brassica oleracea* seedlings. Specifically, we describe the design details of the device, report results of validation tests, and discuss advantages and limitations for its use in investigating the interaction between gravitropism and phototropism on root orientation in seedlings.

## 2. Materials and methods

### *2.1. Device description*

The ROOTROPS device was designed to test the effects of both hypergravity and simulated microgravity and light on root growth and orientation of seedlings. We used seeds of *Brassica oleracea* supplied by Bavicchi S.p.A., Italy (batch n. 181654). Overall, the device was shaped to fit the gondolas of the LDC at ESA's ESTEC in Noordwijk (The Netherlands). A smaller device was developed to be implemented in the large-size Random Positioning Machine (RPM) (Fokker/Dutch Space/EADS, Leiden, the Netherlands) with an inner frame size of 50 × 50 cm.
The basic idea was to construct an easy-to-produce and inexpensive hardware to enable plant experiments in altered gravity. The device consists of two units: the internal one keeps the seedlings in place throughout the experiment, while the external one keeps several internal units in a defined position with respect to the gravity and light directions.

#### *2.1.1 Internal unit: seedling containers*

The internal unit was designed to fulfil the following requirements: a) securing seed position during experiment set-up and avoiding seed displacement due to the hypergravity pull; b) guaranteeing water availability for root growth; c) ensuring root exposure to light.

The ROOTROPS experiment used pregerminated seeds of *B. oleracea*. When the root was approximately 10 mm long, the young seedlings were placed with the root axis parallel to the gravity direction. To avoid seedling detachment and fall from the filter paper, germinated seeds were transplanted in a strip of white filter paper specially folded. More specifically, the strip (13 × 4 cm) was preliminarily punctured in five aligned and uniformly distributed points and folded as shown in Fig. 1a. At the time of experiment assembly, the strips were soaked in deionized water and the germinated seeds were carefully positioned with the root coming out of the holes punctured in the filter paper (Fig. 1b, c).

The seedling container consisted of a disposable plastic Petri dish with a squared shape (12 × 12 × 1.5 cm). We used the cover lid as the lower part of the seedling container and layered it with sized adsorbing paper. In addition, we used black paper (cellulose tissue, 21 g/m$^2$) to enhance contrast for photography. At the time of assembly, we poured 9 ml of deionized water onto the black paper and rolled a small glass-cylinder to remove any possible air bubbles and flat the surface. We then positioned the wet white strip containing pre-germinated seeds on the wet black paper (Fig. 1d). At this point, we inserted the bottom part of the Petri dish to secure both the black paper and the white strip containing the seedlings against possible slipping (Fig.1e). The upper fold of the white strip helped to keep the root tips on the black paper and vertically positioned, avoiding possible displacement during subsequent handling. Finally, we sealed the seedling container with a strip of Parafilm M (Fig. 1f).

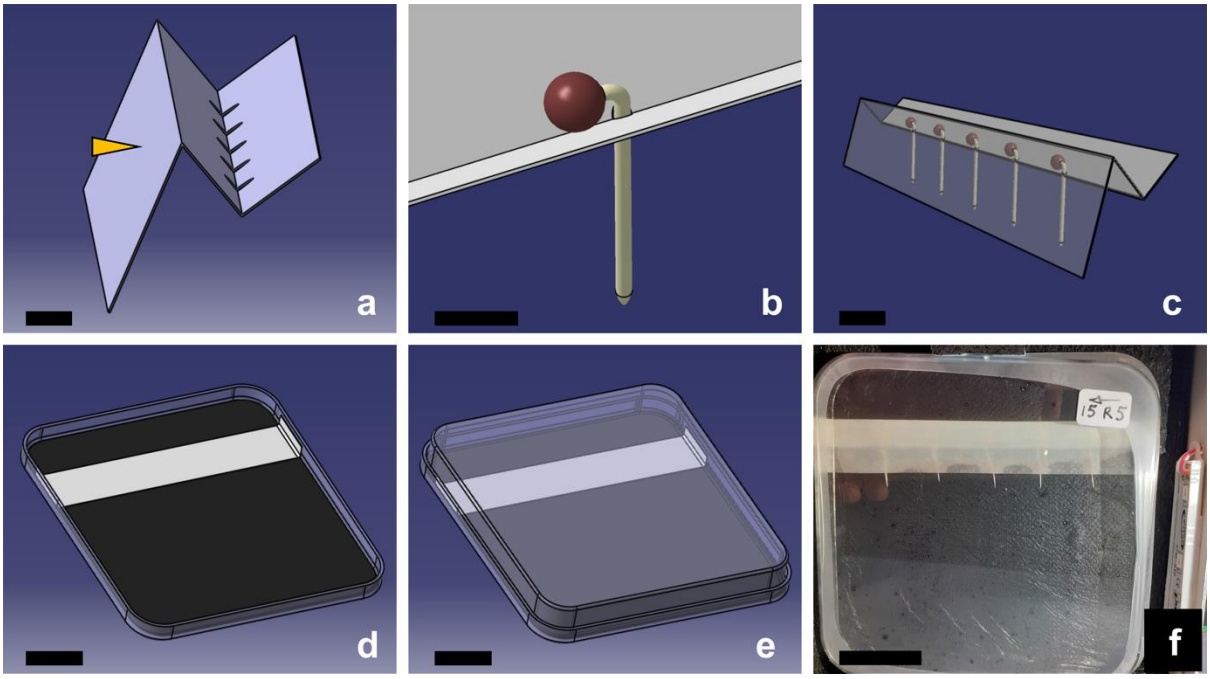


**Figure 1**. ROOTROPS internal unit: a) filter paper strip punctured and folded (the orange arrow points to the front side); b) detail of the pre-germinated seed with the root protruding the filter paper strip; c) filter paper strip and pre-germinated seeds ready to be placed in the Petri dish; d) a Petri dish lid layered with the black filter paper and the white strip holding the seeds; e) Petri dish bottom placed onto the lid to secure the black and white papers; f) photograph of the front view of a fully assembled internal unit. Bar = 5 mm (a, b, c). Bar = 30 mm (d, e, f).

### *2.1.2 External unit: holder box for seedling containers*

The external unit of the ROOTROPS device was designed to fulfil the two following requirements: a) holding several seedling containers in the LDC gondolas and RPM to allow for root growth along the direction of gravity and b) ensuring root exposure to light sources perpendicular (LDC) or random (RPM) to the gravity direction.

The external unit consisted of a multi-slot rack for both the LDC gondolas (30 × 22.5 × 34 cm) and the large-size RPM (17 × 17 × 12 cm), and each slot was sized to contain the internal units in vertical position according to the gravity direction. Each multi-slot rack could house a total of 20 internal units for the LDC gondolas and 5 internal units for the large-size RPM. For the LDC, the internal units were symmetrically distributed in two layers (one on another) and two back-to-back groups (Fig. 2a). For the RPM, we used a configuration of five internal units.

The holder box was made of polystyrene boards (20 mm thickness), a light material that is easy to assemble (Fig. 2b). The two lateral sides of the holder box were covered by aluminum panels, each equipped with LED strips positioned to plug all slot openings (Fig. 2c). Possible leakage of light between adjacent slots was avoided by carefully sealing the polystyrene parts with vinyl glue. Reflections of light were limited by painting in black the polystyrene boards of the holder box. One of the two front sides of each holder box was left uncovered to allow a live monitoring of samples and to record a time-lapse video of seedling growth (Video S1) throughout the experiment run in the LDC (Fig. 2d). For this purpose, we used an USB camera with 180° FoV and 1080p resolution (Model. ELP-USBFHD01M-BL36, Alipu Technology Co., Ltd., Shenzhen, China).

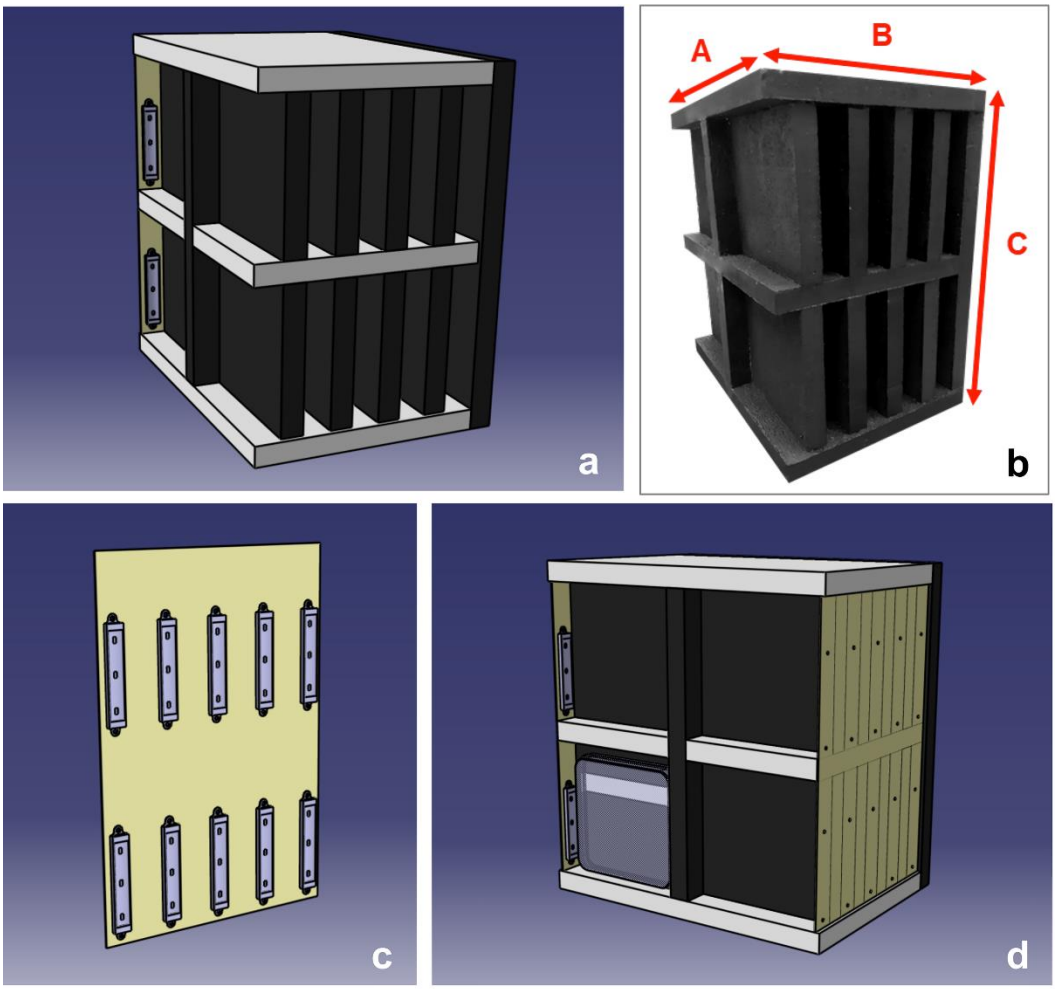


**Figure 2**. ROOTROPS external unit: a) sketch of the holder box with the upper-lower layers and back-to-back slots; b) photograph of the holder box made of black polystyrene (A=30 cm, B=22.5 cm, C=34 cm); c) aluminum panel equipped with LED strips; d) assembled holder box with the uncovered front sides implemented with one internal unit. The 3D images of the holder box and seedling container, and the circuit design of the lighting system are reported in Supplementary Material.

To achieve scientific goals of ROOTROPS, light sources were setup orthogonally relative to the gravity vector (Fig. 3). Four different light conditions were used: white (W), red (R), blue (B), no light (dark, D). The position of the LEDs per each light treatment was randomly assigned on the panel. To determine the emission spectrum of each light source, we used a spectroradiometer (SS-110, Apogee

Instruments Inc.) operative in a wavelength range of 340 to 820 nm. Spectral output of blue, red, and white light are reported in Supplementary Material. The blue and red LED lamps had peak wavelengths of 443 nm and 632 nm, respectively, whereas white light consisted of 25% blue (400 to 500 nm), 53% green (500 to 600 nm), and 21% red (600 to 700 nm). The Photosynthetic Photon Flux Density (PPFD) of the different light sources was equalized by means of dimmers connected to the LED. The light intensity gradient was similar in all light treatments (Fig. 4).

Temperatures were monitored throughout a 24 h run in the W, R, B, and D treatment compartments. The temperature sensors (Escort Mini Temperature Logger) were located in each slot of the holder box at the same distance from the LEDs. An additional temperature sensor was located in the gondola close to the USB camera to measure temperature outside of the holder box. At the end of the run, mean and standard deviation of the recorded temperatures resulted as follow: W = 26.5 ± 0.5 °C; R = 26.2 ± 0.6 °C, B = 26.8 ± 0.5 °C, D = 26.0 ± 0.6 °C; Outside the box = 26.2 ± 0.5 °C. Ambient temperature in the laboratory during the run was 26.0 ± 0.5 °C.

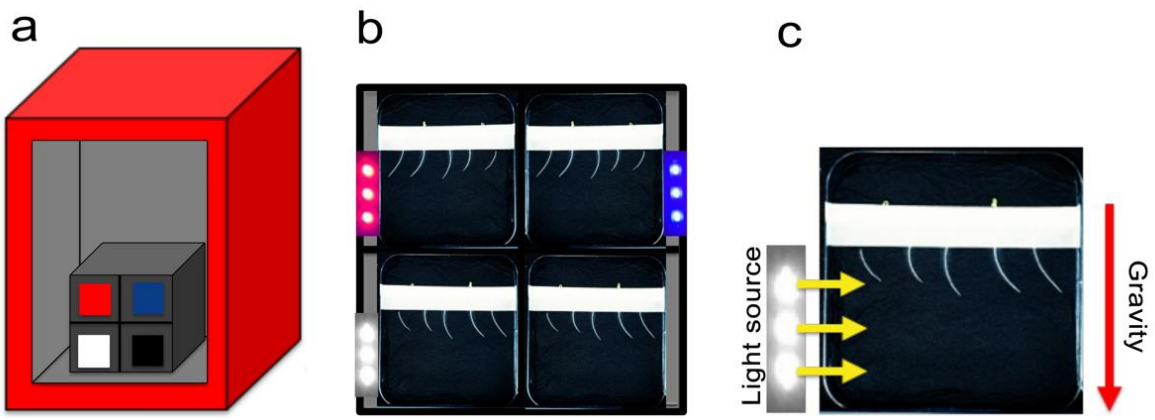


**Figure 3**. Schematic representation of the ROOTROPS setup. a) gondola of the Large Diameter Centrifuge implemented with the ROOTROPS device, b) diagram of the frontal view of the ROOTROPS external unit with light sources and four internal units, c) diagram of the configuration of a single internal unit with respect to light and gravity directions.

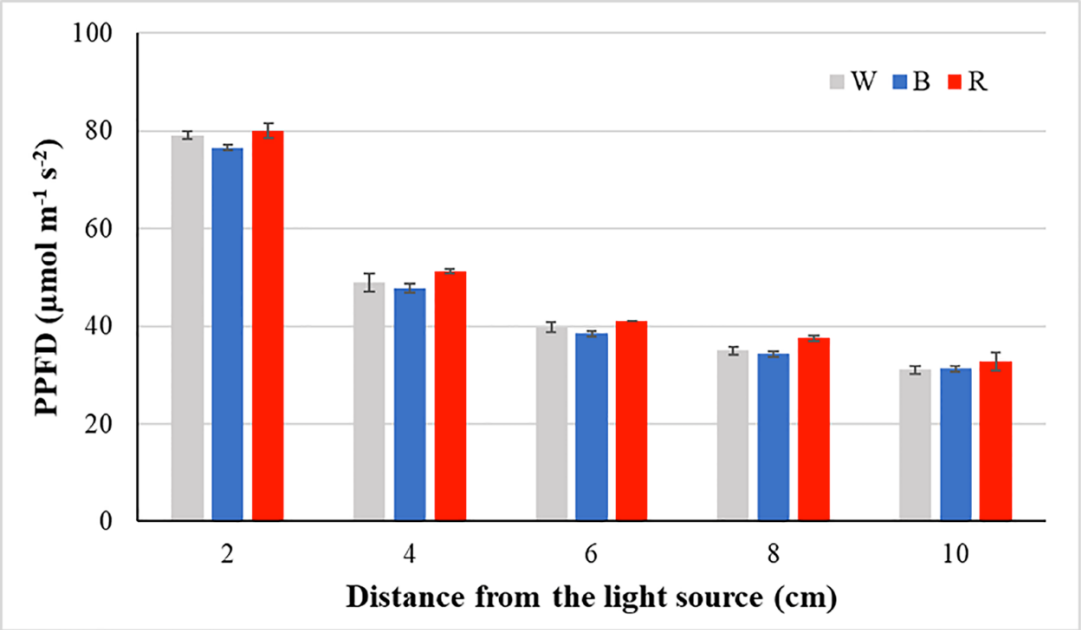


**Figure 4**. Light intensity measured at different distances from the light sources (W=white, B=blue, R=red). Means of PPFD of the different light sources at the same distance were not significantly different ($P$>0.05).

Four different hypergravity treatments were used: 20*g*, 15*g*, 10*g*, 5*g*. Two gondolas per each treatment were utilized. The overall set up was defined to have the gravity value closer to nominal at the center of the holder boxes. The range of variation for hypergravity and simulated microgravity values is reported in Table 1. During each experiment run, an additional 1*g* control treatment was set up used in the holder boxes with the seedling containers in a static gondola placed in the same room as the centrifuge to have the same environmental conditions.

**Table 1:** Range of variation for the gravity treatments in the Large Diameter Centrifuge (LDC) and Random Positioning Machine (RPM). Considering the size of the ROOTRPS device, real gravity values were calculated according to the possible position of the seedlings

within the volume space of the external/internal units (lateral or distal from the radial line of rotation) and the swing-out angle of the gondola at different speeds.

| | Hypergravity (LDC) *g* | | | | Simulated microgravity (RPM) *g* |
|---|---|---|---|---|---|
| Nominal | 5 | 10 | 15 | 20 | |
| At device center | 5.01 | 10.00 | 15.04 | 19.08 | |
| Minimum | 4.82 | 9.64 | 14.17 | 18.97 | |
| Maximum | 5.25 | 10.35 | 15.90 | 19.75 | |
| Center of rotation | | | | | µg |
| At perimeter of rotation | | | | | $<10^{-4}$ |

For the RPM tests, we used a configuration with five internal units only. Consecutive tests were performed to reach the number of replicates as with the LDC. The center of this assembly was placed in the center of rotation of the large size RPM in order to reduce the residual centrifugal force due to rotation (Hasenstein & van Loon, 2015; van Loon, 2007). In this configuration, all seedlings were located less than 10 cm from the center of rotation. Running at a maximum random speed of 60°/s, the residual acceleration was less than $10^{-4}g$ (Table 1). The system was also set to random direction and interval.

Before the experiment activation, we inserted all seedling containers in the holder box and finally assembled the entire system in the LDC (Fig. 5 a, b) and the RPM (Fig. 5 c).


**Figure 5**. ROOTROPS device in the ESA-LDC: a) a gondola with a holder box implemented with LEDs and 20 seedling containers; b) a computer display of the LDC system showing a real-time image of the samples in the gondola recorded by the internal camera while running at 20*g*; c) the large-size RPM with the holder box implemented with LEDs and 5 seedling containers.

### *2.2 Device validation tests*

For the validation tests, the ROOTROPS device was assembled and set up as for the scientific experiment. The system was tested under increasing hypergravity levels up to 20*g* for periods of 24 h. At the end of the runs, we evaluated the possible effects of hypergravity on the system by visual observations and specific measurements.

We carefully inspected the external units to check possible damage including breakage of the polystyrene panels or loosening of the LED strips and other electrical parts.

We checked the seedling containers to identify the possible occurrence of seedlings detached from the original position. We also used the images recorded for the time-lapse video to measure possible slipping of the white strip holding the seedlings. Specifically, for each seedling container, we compared the position of the white strip on images recorded before and after 24 h of hypergravity treatment. On each set of coupled images, we selected one easily identifiable point on the white strip and measured its vertical distance to the bottom edge of the seedling container by using

ImageJ v1.53e software (Schneider et al., 2012). On each image, the distance was averaged based on five measurements to decrease estimation uncertainty.

We measured the effect of the centrifugal force on the water retention capacity of the black paper aimed at furnishing a uniform water availability to the developing roots. At the end of each run, we halved the black paper of each container by cutting it perpendicularly to the gravity direction. To evaluate the water gradient within each container, we then compared water content of each half measured as the difference between fresh and dry weight (50 °C for 24 h) of the paper.

At the end of the test, we also verified possible visible damages on the external appearance of the seedlings. Moreover, to evaluate the device suitability with the biological system, we counted the number of seedlings whose roots developed on the black paper surface in comparison to those that have pierced the paper growing hidden between the paper and the Petri dish lid.

### *2.3 Statistics*

Data from the validation tests were statistically analyzed using Excel ver. 16 (Microsoft Corp.) and SPSS Statistics ver. 26 (IBM Corp.). The normality of the datasets was assessed by the Kolmogorov-Smirnov's and Shapiro-Wilk's tests. For both the seedling set-up slipping test and the water gradient estimation test, data obtained were normally distributed, and the homogeneity of variance was verified using the Levene's Test. We therefore compared the means applying a one-way analysis of variance (ANOVA) followed by Tuckey's post-hoc test ($P<0.05$). The number of roots under the black paper was limited to a few samples, and data significantly deviate from a normal distribution. In such a case, we applied a Pearson Chi-square test of independence to verify the hypothesis that the number of hidden roots was equally distributed in the six gravity treatments.

### 3. Results

At the end of the 24-h runs, we visually inspected the external units of the ROOTROPS device and observed neither breakage of the polystyrene panels nor loosening of the LED strips and other electrical parts in all boxes subjected to any gravity treatment. We also inspected the internal seedling containers taking note of possible slipping of paper parts or seedling detachment. We observed water leakage from the bottom of the seedling containers; the phenomenon was more evident in highest hypergravity treatments. The assembled system was unaltered, with no sign of shift or displacement even when subjected to the highest hypergravity treatments or the RPM movements. These results were also confirmed by data from the image analysis aimed at comparing the distance from the white strip to the bottom edge of the seedling containers recorded before and at the end of the runs. Results of the ANOVA consistently showed no significant difference ($P$>0.05) in the position of paper strip holding the seedlings at any gravity level (Fig. 6).

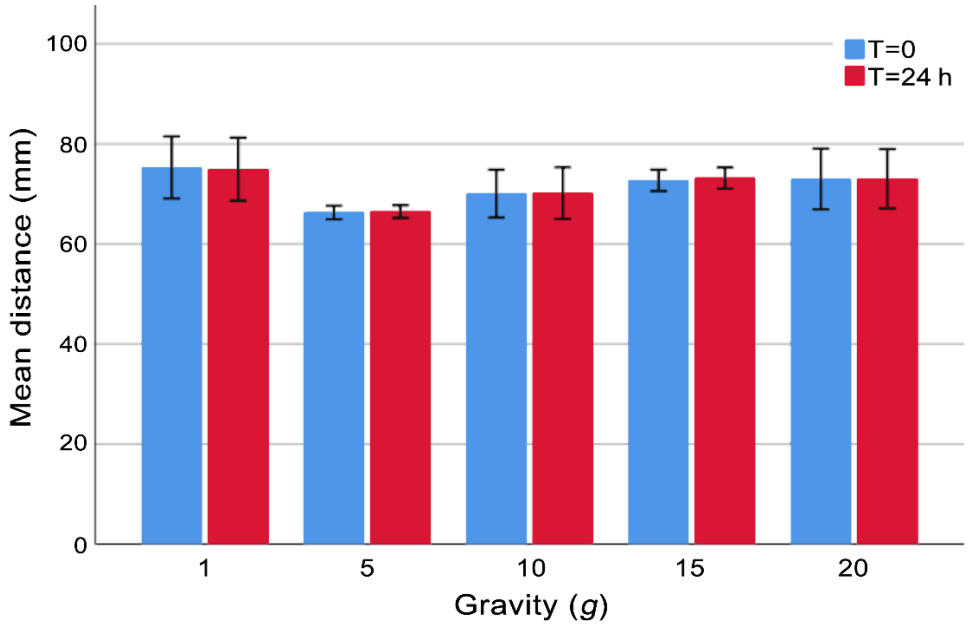


**Figure 6**. Position of the white strip in the seedling container measured before (T=0) and at the end of the experiment run (T=24 h). Each value shows the mean and standard deviation

of 5 replications. The ANOVA showed that mean distances were not significantly different for any gravity level ($P$>0.05).

The water gradient was expected to be determined by the gravity level. Statistical analysis of the data by gravity treatments confirmed that water was similarly distributed in the two halves of the black paper only in the seedling containers on the RPM ($P$>0.05). When subjected to a unidirectional gravity vector, the quantity of water retained by the paper in the upper part of the container was lower than that in the bottom part. Specifically, the difference was significant ($P$<0.01) at 1$g$, and highly significant ($P$<0.001) from 5$g$ and higher (Fig. 7). Moreover, such differences linearly increased with the hypergravity level ($R^2$=0.79, $P$<0.001).

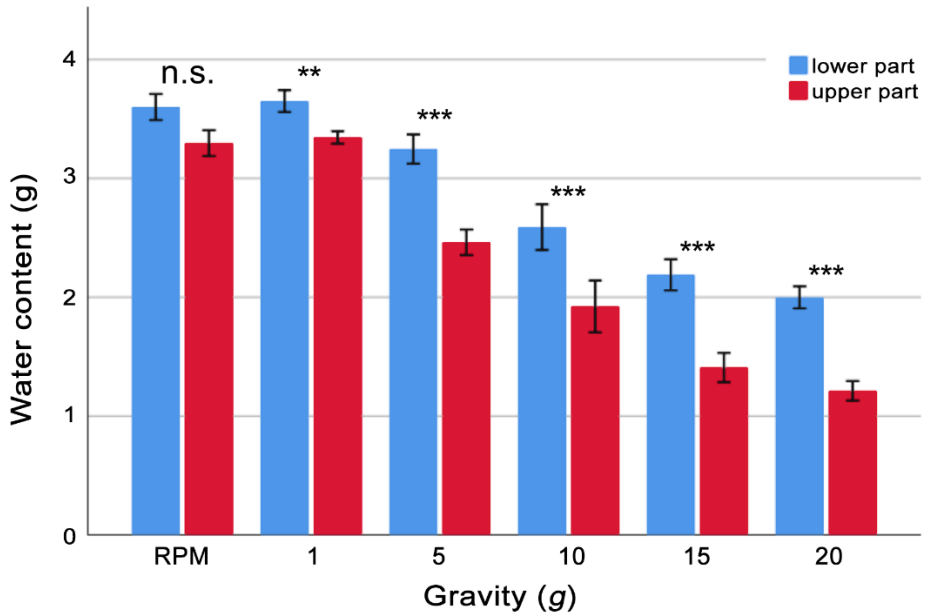


**Figure 7**. Water retained by the black paper in seedling containers at the end of the experiment: difference between fresh and dry weight of the lower and upper halves. Each value shows the mean and standard deviation of 8 replications. Within the gravity levels, the difference was statistically not significant (n.s.=$P$>0.05) at simulated microgravity, significant (**=$P$<0.01) at 1g, and highly significant (***=$P$<0.001) from 5g and higher.

Focusing on possible damages to the seedlings, their external appearance did not show visible sign of injury. Seedlings had robust growth/morphology as shown in the time-lapse video in Supplementary Material, and the effects of light and gravity on root tip orientation were clearly detectable. Roots grew attached to the black paper surface and the resulting color contrast between the white roots and the black paper simplified the subsequent image analyses required to achieve the scientific aims of the ROOTROPS experiment.

In a few cases, we observed that the root had pierced the black paper, growing further hidden behind it (Fig. 8a). Such a phenomenon occurred in 5.3% of the developed seedlings (63 out of a total of 1188). Results of the Chi-squared test showed that the distribution of the hidden roots (Fig. 8b) was independent of the gravity treatment ($\chi^2$=6.619, *P*=0.25).

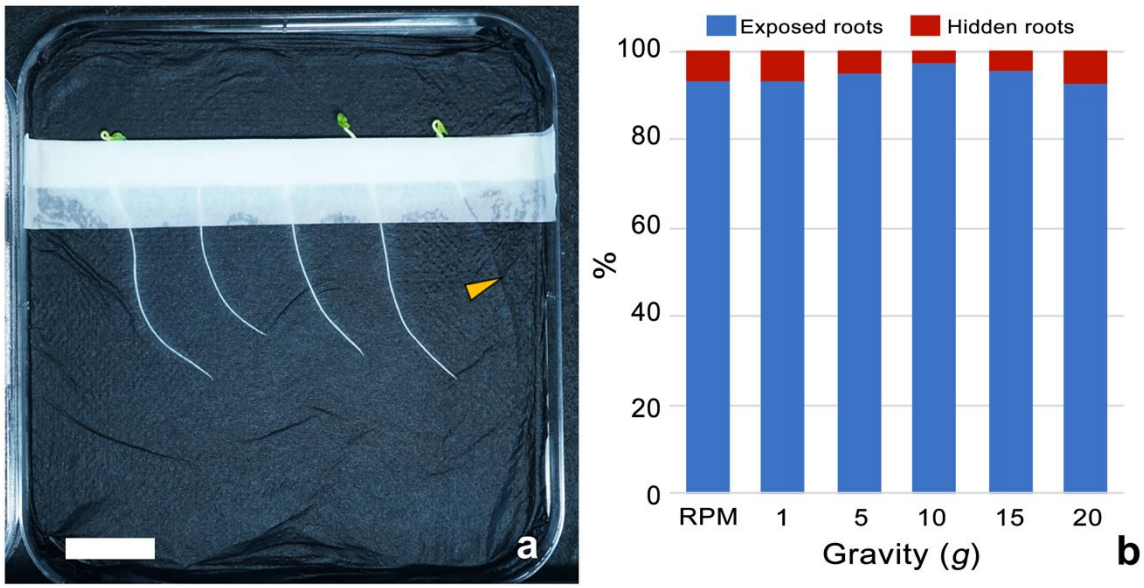


**Figure 8**. Images of roots at the end of the ROOTROPS experiment: a) Seedling container with the grown seedlings; the yellow arrow points to the root growing behind the black paper; b) percentages of exposed and hidden roots per each gravity level on a total of 1188 seedlings. Differences were statistically not different ($\chi^2$=6.619, *P*=0.25). Bar = 20 mm.

## 4. Discussion

The ROOTROPS experiment was successfully performed in December 2019 and February 2020 at the ESTEC laboratories, and technical and scientific goals were fully achieved, showing that the device to study root tropisms in the LDC was functional and effective.

In the framework of plant cultivation in space (on orbital platforms, the Moon, and Mars), a better understanding of the interactions among tropisms in altered gravity conditions is necessary (Muthert et al., 2020). On Earth, the role of gravitropism in orienting root growth into the soil is well established (Darwin & Darwin, 1880; Kiss, 2000; Nakamura et al., 2019). In contrast, the evolutionary role of root phototropism is more controversial and generally associated to light penetration in the soil (Mandoli et al., 1990; Kiss et al., 2003). Research on root phototropism has been relatively limited after systematic studies by Schaefer (1911) and Hubert and Funke (1937), but it is currently gaining significant attention thanks to the possibility of performing experiments in space and to the development of LED lighting systems for plant cultivation (Gómez & Izzo, 2018; Izzo & Aronne, 2021). Otherwise, attempts to better reproduce in the lab the conditions of plant culture in nature, in terms of the different illumination of the roots and of the aerial parts, have been done (Silva-Navas et al., 2015).

Root phototropism is weak compared to gravitropic responses (Kiss et al., 2003), and, as well as for other tropisms, numerous experiments have been performed reducing the dominance of gravitropism by subjecting the plants to the effect of simulated microgravity (on Earth) or real microgravity (in space) (e.g. Kiss et al., 2012; Vandenbrink et al., 2016). However, no studies have been conducted so far to evaluate root phototropism in hypergravity conditions, possibly due to the unavailability of suitable devices. The ROOTROPS experiment was conceived in this scenario with the aim of expanding phototropism research to hypergravity. Therefore, it was necessary to design and develop an easy-to-produce and inexpensive device to study phototropism/gravitropism interactions in seedlings.

To achieve the scientific goals, seedling roots had to be free to grow not only along the gravity direction but also toward (or away from) the light sources that were placed orthogonally relative to the gravity vector. Such a requirement was challenging to accomplish avoiding at the same time any seedling movement (such as detachment from the growth substrate, rolling, slipping). In the ROOTROPS device, the peculiar folding of the filter-paper strip proved to be an appropriate solution. The validation tests showed that all seedlings remained in place and free to direct the growth of both root and shoot apices while subjected to all gravity treatments, up to 20*g*. The idea of rolling out the black paper on the lid of the Petri dish and use the bottom to clamp both the black and the white paper strips was successful as well.

The use of filter paper layering the Petri dish is a very common method for seed germination and early-stage seedling development (Baskin & Baskin, 2014). The horizontal position of the dish allows a uniform distribution of the water in the filter paper. The amount of water we poured to hydrate the substrate resulted in a greater quantity than was holdable by the paper. We expected that the vertical position of the ROOTROPS seedling container would cause a gradient in the water retained by the black filter paper due to the competition between the capillary force of the tissue fibers and the weight of the water due to gravity. Data on water gradient confirmed this hypothesis showing that the difference was proportional to the hypergravity treatment. However, this phenomenon did not exert visible damage to the seedlings but must be considered in the context of discussing the biological results.

Previous experiments showed that the root response to light can be different according to different light wavelengths and specifically in response to blue versus red light (Millar et al., 2010; Silva-Navas et al., 2015; Sindelar et al., 2014; Vandenbrink et al., 2016) and that root development is mediated by light intensity (Kumari et al., 2019). Up to 20 different light treatments can be tested simultaneously in each ROOTROPS device and numerous combinations of light spectra and intensities can be easily achieved by changing the LEDs emission spectrum and adjusting PPFDs using dimmers. However, for the design of experiments using this device it is important to note that light intensity can vary

greatly based on the distance from the light source, but this is a characteristic of the LEDs which has been already highlighted for other hardware including the VEGGIE growth chamber on the ISS (Massa et al., 2016).

Paper piercing by the developing roots was an unexpected phenomenon. The frequency was quite limited and was trigged only when a small wrinkling was left on the black paper surface. We hypothesize that the issue can be reduced, up to elimination, by a more complete paper flattening at the time of the experiment's implementation.

The ROOTROPS device was effective in keeping seedlings in position in hypergravity and also on the RPM. The water gradient did not occur in the RPM, suggesting that this device can be further used not only as control test of the hypergravity experiments as for ROOTROPS, but also to investigate the effects of light quality and intensity in simulated microgravity conditions.

Plant experiments in microgravity have contributed significantly to our understanding of plant perception and reaction to gravity (Ruyters & Braun, 2014; Vandenbrink & Kiss, 2016). By attenuating the gravity stimulus, the effect of other stimuli (such as light, water, chemicals) can be relatively enhanced (Muthert et al., 2020), and hypotheses aimed at clarifying specific mechanisms of gravitropism can be tested. To study the effects of gravity on plants, research in true microgravity is by far the most expensive and complex method in terms of both cost and time (Böhmer & Schleiff, 2019). This fact compels researchers to use ground-based devices, while accepting to deal with their constraints. Clinostats (Dedolph et al., 1967; Hoson et al., 1997) and Random Positioning Machines (Borst & van Loon, 2009; van Loon, 2007) are the most frequently used (Kiss et al., 2019). However, in some cases, hypergravity can also be utilized to investigate the effects of microgravity (Herranz et al., 2013; van Loon, 2016).

Using a novel approach, the ROOTROPS experiment was not only focused on simulated microgravity but also on investigating root responses to different light wavelengths while enhancing the gravity stimulus. In this circumstance, seedlings of *B. oleracea* were subjected to high levels of hypergravity (up to 20*g*) for extended

periods, and thereby enhancing the risk of seedling detachment and substrate slipping. Notwithstanding the demanding requirements, ROOTROPS device was reliable in achieving scientific goals and the few weaknesses were evaluated as acceptable.

In summary, the ROOTROPS device is an easy-to-produce and inexpensive (ground-based) apparatus that enables investigation of the effect of light stimuli on root orientation, retaining seedlings in place while being exposed to altered gravity from simulated microgravity to centrifugation up to 20*g*. The polystyrene holder box can be reused many times to run new experiments or to replicate the same. The device will be used in future experiments aimed at improving our understanding of phototropism-gravitropism interactions and may also be considered as basic concept for hardware for future space experiments.

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# Appendix 1B

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## Interaction of gravitropism and phototropism in roots of *Brassica oleracea*

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# Interaction of gravitropism and phototropism in roots of *Brassica oleracea*


## Abstract

Gravitropism and phototropism play a primary role in orienting root growth. Tropistic responses of roots mediated by gravity and light have been extensively investigated, and a complex mutual interaction occurs between these two tropisms. To date, most studies have been conducted in 1*g*, microgravity, or simulated microgravity, whereas no studies investigated root phototropism in hypergravity. Therefore, we studied the effects of several gravity treatments with those of different light wavelengths on root growth orientation. Here, we report growth and curvature of *Brassica oleracea* roots under different *g* levels, from simulated microgravity up to 20*g*, and unilateral illumination with different spectral treatments provided by light emitting diodes. Microgravity was simulated with a random positioning machine whereas hypergravity conditions were obtained using the Large Diameter Centrifuge at the laboratories of the European Space Agency in the Netherlands. Four light treatments (white light, blue light, red light, and dark) were used in this study. Overall, roots of seedlings grown in the dark were longer than those developed under unilateral light treatments, regardless of the gravity level. Unilateral blue light or white light stimulated a negative phototropism of roots under all *g* levels, and root curvature was not affected by either hypergravity or simulated microgravity compared to 1*g*. Results also confirmed previous findings on the effect of light intensity on root curvature and highlighted the relevance of blue-light photon flux density in root phototropism. Roots illuminated with red light showed a weak curvature in simulated microgravity but not in hypergravity. Moreover, root curvature under red light was similar to dark-grown roots in all *g* levels, suggesting a possible involvement of surface-dependent phenomena in root skewing under either red light or dark conditions. Further studies can confirm phototropic responses of *B. oleracea* in the weightless environment of orbiting spacecraft. Nevertheless, according to our

findings, directional lighting represents an effective stimulus to guide root growth in a wide range of gravity conditions.

**1. Introduction**

Gravitropism and phototropism are directional growth responses of plant organs to gravity and light, respectively. Numerous studies have focused on plant tropisms since those of Darwin who pioneered modern research on gravitropism and phototropism (Darwin and Darwin, 1880). During the water-to-land transition, seed plants evolved rapid gravitropic responses of roots which facilitated the adaptation to the terrestrial environment (Zhang et al., 2019). Generally, to harvest light while anchoring to the substrate, plants orient shoots toward the light (positive phototropism) and away from the gravity vector (negative gravitropism) and, conversely, orient roots into the soil, away from light (negative phototropism) and toward the direction of gravity (positive gravitropism) (Gilroy, 2008). Plant roots have also evolved several other tropisms (e.g., hydrotropism, chemotropism, thigmotropism, magnetotropism, electrotropism, and phonotropism) to orient their growth according to a wide range of environmental stimuli, and phototropism is among the most studied together with gravitropism (Muthert et al., 2020).

As already reported in the late 1800s by Darwin and Darwin (1880), the root tip acts as a “brain” in governing organ orientation by actively perceiving and responding to tropistic stimuli. To date, four different zones with distinct cell populations in the root tip of *Arabidopsis thaliana* have been reported (Verbelen et al., 2006; Baluška et al., 2010), as well as the location of all known sensor and action regions involved in root tropisms (Muthert et al., 2020). Specifically, sensors for root gravitropism and phototropism are located in the root cap and in the elongation zone, whereas action regions for root gravitropism and phototropism are the transition zone and the elongation zone, respectively (Blancaflor et al., 1998; Briggs and Christie, 2002; Mullen et al., 2002; Sakamoto and Briggs, 2002; Wolverton et al., 2002; Kiss et al., 2003). Although gravitropism and phototropism have completely different sensors for tropistic stimuli (i.e., statoliths for gravity, phototropins and phytochromes for light), their transduction pathways exhibit a complex interaction in the control of organ orientation (Correll and Kiss, 2002). According to the Cholodny-Went theory,

auxin accumulation in the root tip inhibits cell elongation at the lower side of the root, causing the root to bend in the direction of gravity (Geisler et al., 2014). Similarly, root phototropism acts through differential auxin distribution mediated by asymmetrical distribution of PIN FORMED 2 (PIN2) proteins upon phototropin 1 (phot1) activation (Pedmale et al., 2010; Zhang et al., 2014). In this model, NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3) influences PIN2 distribution and is a point of interaction for gravitropic and phototropic signaling (Wan et al., 2012). Nevertheless, a study on *A. thaliana* by Kimura et al. (2018) suggests that the asymmetrical increase in auxin on the illuminated side of the root is a gravitropic reaction following the initial phototropic bending. It is also noteworthy that light can operate not only as a tropistic signal in plants, but possibly also as a tonic (turgor) signal affecting graviresponse as has been documented in fungi (Galland, 1998; Grolig et al., 2000).

Interaction of root gravitropism and phototropism also involves the phytochrome-dependent regulation of PHYTOCHROME KINASE SUBSTRATE 1 (PKS1) which negatively regulates gravitropism and contributes to phototropin-mediated phototropism (Boccalandro et al., 2008). Phytochromes are also directly involved in the regulation of root phototropism and the different responses of phytochrome A and phytochrome B allow the integration of multiple environmental stimuli including gravity (Kiss et al., 2003). Phytochromes are involved in several responses of plants and their photosensory activity relies on a reversible switching between inactive and active form mediated by red and far-red light (Quail, 2002). However, phytochromes are photoreceptors that also absorb blue light, which can influence the phytochrome photoequilibrium with implications in plant photomorphogenesis (Smith, 2000; Meng and Runkle, 2017; Kong et al., 2018; Kong et al., 2019), possibly affecting also phototropic responses.

Although positive gravitropism of roots is ubiquitous in higher plants (e.g., Ge and Chen, 2016), root phototropic responses exhibit considerable variability. Systematic studies on a total of about 300 species reported that about a half did not react to unilateral white light, whereas the other half showed a negative phototropism of

roots, and a few species displayed a positive response (Schaefer, 1911; Hubert and Funke, 1937). More recently, tropism research has focused on disentangling the molecular pathways in *A. thaliana* (Muthert et al., 2020), whose roots show negative phototropic responses to white and blue light like most species of the Brassicaceae family tested by Schaefer (1911) and Hubert and Funke (1937). To date, it is known that *A. thaliana* roots can exhibit negative and positive phototropism to blue and red light respectively (Kiss et al., 2012), but also that phototropic responses may not be predictable in altered gravity as in the case of the blue-light positive phototropism discovered in microgravity (Vandenbrink et al., 2016). Specifically, the blue-light positive phototropism of roots was only detectable at gravity levels below 0.3 g and pre-treatment with 1 h of red light enhanced the response (Vandenbrink et al., 2016). Similarly, other tropisms have been revealed in microgravity such as the chemotropism of *Daucus carota* roots toward disodium phosphate (Izzo et al., 2019), and the red-light phototropism of *A. thaliana* hypocotyls (Millar et al., 2010; Kiss et al., 2012), indicating that the relatively strong gravitropic responses typically mask other tropisms.

According to the vector hypothesis, the actual degree of root bending in *A. thaliana* depends on the phototropic response and a counteracting gravitropic response, resulting as the sum of gravity and light vectors (Okada and Shimura, 1992; Vitha et al., 2000). Phototropic responses involve deviation of the growth direction from the gravity vector and generate a gravitational stimulus that partially counteracts phototropism. Consequently, mutants with deficient gravitropic response show enhanced root phototropism (Okada and Shimura, 1992; Vitha et al., 2000). Similarly, it has been shown that attenuating the effects of gravity or using mutants that are impaired in gravisensing, the red-light positive phototropism of roots can be revealed (Ruppel et al., 2001; Kiss et al., 2003; Kiss et al., 2012). Tropism research has also shown that both positive and negative phototropic responses of *A. thaliana* roots are dependent on photon flux density at very low values and saturate at about 10 µmol $m^{-2}$ $sec^{-1}$, whereas responses are constant at higher photon flux densities for both blue- and red-light phototropism (Sakai et al., 2000; Kiss et al., 2003).

Overall, these findings suggest that changes in the quality and magnitude of light and gravity stimuli can influence the interaction of gravitropism and phototropism in roots and that tropistic responses can vary strongly between species. Despite the extensive research conducted under gravity conditions ranging from microgravity to 1*g*, few experiments investigated root tropisms in hypergravity (Muthert et al., 2020). It has been shown that an acceleration of 5*g* is required for the restoration of root gravitropism in starchless mutants of Arabidopsis, and this response was associated with increased sedimentation of plastids (Fitzelle and Kiss, 2001). Still, to date, no studies investigated the interaction of gravitropism and phototropism of roots under hypergravity conditions.

Thus, it remains to be verified whether the same relationship between root gravitropism and phototropism holds when increasing the magnitude of gravity using different light spectra. Furthermore, it is necessary to expand the knowledge on plant tropisms to other species, particularly considering candidate crops for cultivation in space where gravity conditions can alter the interaction between the different tropisms (Izzo et al., 2021a). Recently, the increasing possibility of performing experiments in altered gravity, together with the development of narrow-band Light Emitting Diodes (LEDs), is paving the way toward a better understanding of gravitropism and phototropism interaction (Borst and van Loon, 2009; Gómez and Izzo, 2018).

In this study, we tested the hypothesis that changes in light quality and magnitude of gravity can influence the net effectiveness of root gravitropism and phototropism of *Brassica oleracea* seedlings. We analyzed growth and curvature of roots under different *g* levels, from simulated microgravity up to 20*g*, in combination with different spectral treatments provided by LEDs and dark conditions.

## 2. Materials and methods

### *2.1. Experimental design and facilities*

The experiment was performed using the Ground Based Facilities at the European

Science and Technology Center in Noordwijk (NL) (Frett et al., 2016) in the framework of the ROOTROPS project funded by the European Space Agency. The experiment consisted of two runs, each including 6 gravity levels (1*g*, 5*g*, 10*g*, 15*g*, 20*g*, and simulated microgravity) and 4 light treatments (white light, blue light, red light, and dark). Conditions of 1*g* and hypergravity were obtained within gondolas of the Large Diameter Centrifuge (LDC) (van Loon et al., 2008), whereas a Random Positioning Machine (RPM) (Fokker / Dutch Space / EADS, Leiden, the Netherlands) was used to simulate microgravity. The experiment was performed using square Petri dishes (12 × 12 × 1.5 cm) as seedling experimental container (EC) and specifically developed hardware to hold the ECs (Aronne et al., 2022). The external hardware consisted of a multi-slot box with an adjustable LED system to provide a stable housing for the ECs, gravity direction according to the seedling root/shoot axis and unilateral light treatments perpendicular to the gravity vector (Figure 1A). Each box housed five ECs per light treatment for a total of 20 ECs which were randomly distributed within the box.

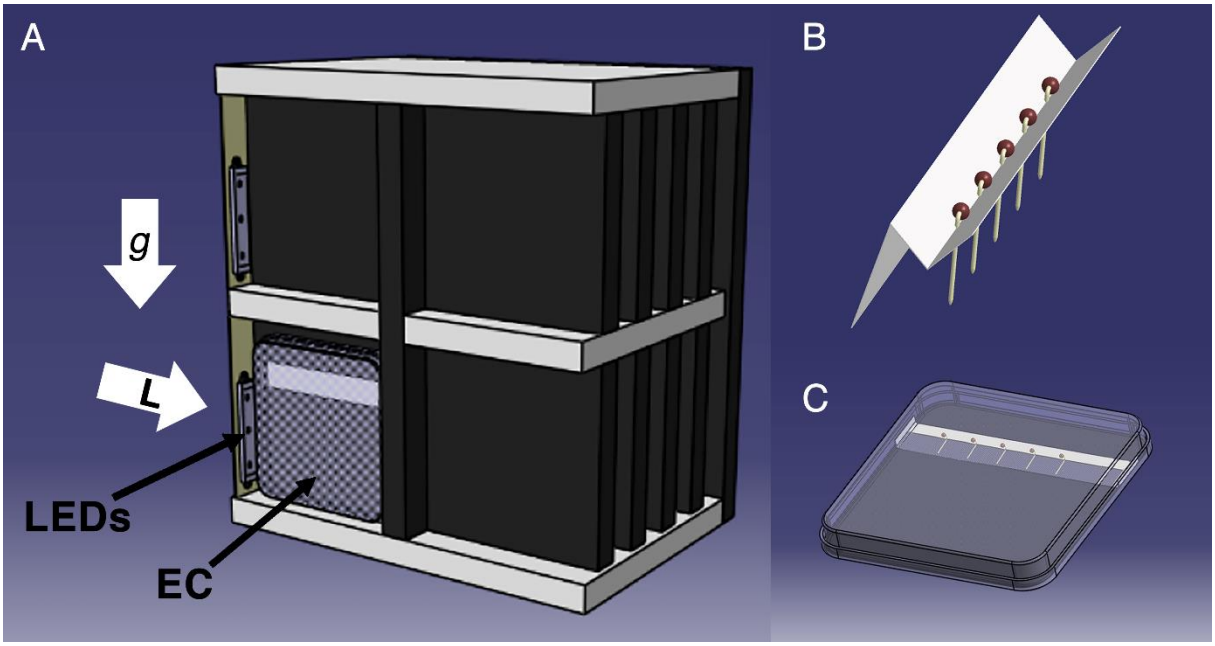


**Figure 1.** Experimental device used for the ROOTROPS experiment. A) multi-slot box with light emitting diodes (LEDs) and the experimental container (EC). White arrows labeled *g* and L indicate the direction of gravity and light vectors, respectively; B) strip of white filter

paper punctured and folded to hold the seedlings; C) experimental container with five seedlings (Aronne et al., 2022).

*2.2. Plant material and EC setup*

Seeds of *B. oleracea* (Bavicchi S.p.A., Italy, batch n. 181654) were surface sterilized in 3% (v/v) sodium hypochlorite/water solution for 5 min and then rinsed with sterile water. Seeds were subsequently germinated on wet filter paper (cellulose; 67 g/m$^2$) in a growth chamber at 26 °C under continuous fluorescent white light for 24 h. During germination, seeds were placed within Petri dishes on a 45° inclined plane to facilitate a straight downward protrusion of roots.

The ECs were lined with black paper (cellulose; 21 g/m$^2$) which was successively wetted with deionized water. Seedlings with a root length of approximately 10 mm were placed in the ECs using a strip of filter paper punctured in five aligned points and folded to form a pocket aimed to insert the roots and anchor the seedlings (Figure 1B). Five seedlings of *B. oleracea* were then placed within each EC (Figure 1C) for a total of 1200 seedlings tested during the two experiment runs (5 seedlings × 5 ECs × 4 light treatments × 6 gravity levels × 2 experiment runs). The ECs with the seedlings were then placed within the multi-slot box and kept in vertical position for 12 h under dark conditions to promote root anchoring to the black paper. Each experiment run was then performed at an ambient temperature of 26 °C for 24 h and was monitored by means of internal cameras.

*2.3. Gravity treatments*

Four different hypergravity levels were tested using the gondolas of the LDC. Overall set up of the gondolas was defined to achieve the nominal gravity values, namely 5*g*, 10*g*, 15*g*, and 20*g*, at the center of the holder boxes. The 1*g* control treatment was set up using a static gondola placed in the same room as the centrifuge to have the same environmental conditions.

For simulated microgravity, all seedlings were located less than 10 cm from the

center of rotation of the RPM to reduce residual centrifugal force due to rotation (van Loon, 2007; Hasenstein et al., 2015). Running at a maximum random speed of 60°/s the residual acceleration was less than $10^{-4}g$. The system was also set to random direction and interval.

*2.4. Light treatments*

Four light treatments were tested in this study: white light, blue light, red light, and dark conditions. A spectroradiometer (SS-110, Apogee Instruments Inc.) was used to determine the emission spectrum of each LED source in the range of 340 to 820 nm and to generate a light-intensity map within the EC for each light treatment (Figure 2). The blue and red LED lamps had peak wavelengths of 443 nm and 632 nm, respectively, whereas white light provided a broad spectrum consisting of 25% blue (400 to 500 nm), 53% green (500 to 600 nm), and 21% red (600 to 700 nm) (Figure 2).

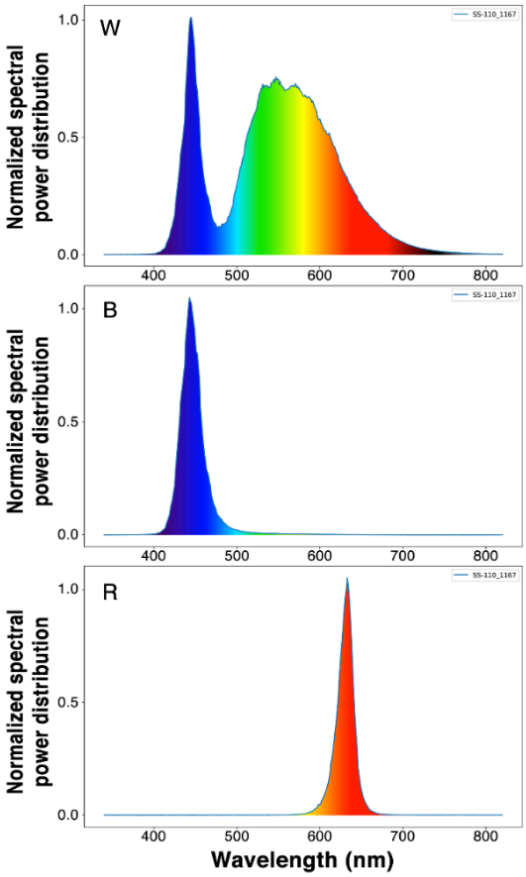


**Figure 2.** Normalized spectral power distribution of the light-emitting diode lamps used in this study: (W) white light; (B) blue light; (R) red light. Spectral scans were recorded at 5 cm distance from the light sources with a spectroradiometer.

Light intensity was controlled using dimmers connected to the LED light sources. Light treatments using white, blue, and red LEDs had an average photon flux density (PFD) of ≈ 80, 50, 40, 35, and 30 µmol $m^{-2}$ $s^{-1}$ at 2, 4, 6, 8, and 10 cm distance from the light source, respectively (Figure 3). Dark conditions were assured by wrapping ECs with aluminum foil and the light mixing between spectral treatments was avoided separating the ECs with a black foam. To test root phototropic responses, unilateral light treatments were provided by LEDs placed on the side of the ECs with light direction perpendicular to the direction of gravity (Figure 1 and Figure 3). The configuration of the light source and the EC determined a range of light intensity decreasing from ≈ 80 µmol $m^{-2}$ $s^{-1}$ (seedlings placed at 2 cm distance from the

LEDs) to ≈30 µmol $m^{-2}$ $s^{-1}$ (seedlings placed at 10 cm distance from the LEDs) for all light treatments (Figure 3).

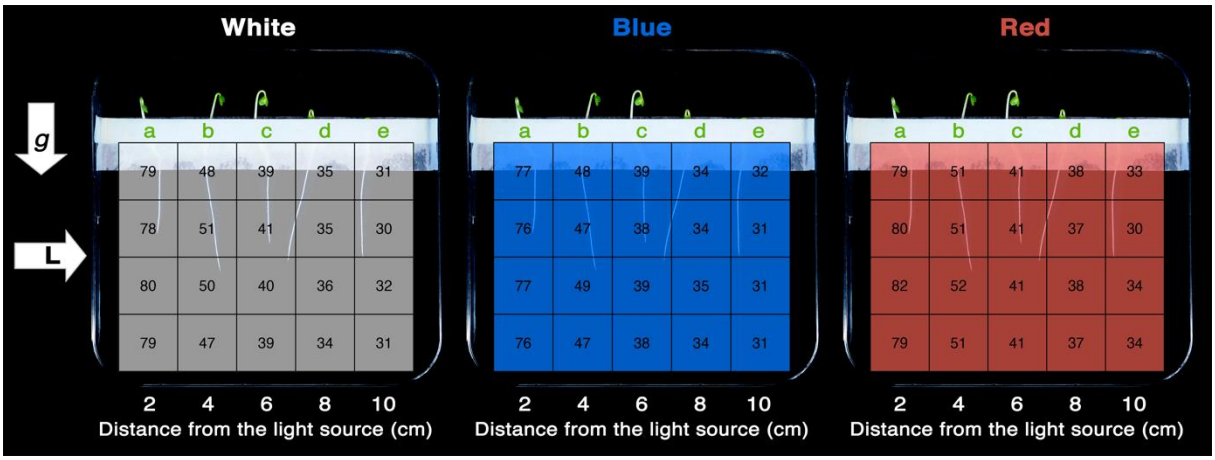


**Figure 3.** Light-intensity map for white, blue, and red LEDs within square Petri dish (12 × 12 cm) used in this study. The values reported in the tables refer to the photon flux density (µmol $m^{-2}$ $s^{-1}$). Green letters indicate the different positions of seedlings according to their distance from the light source (a = 2 cm; b = 4 cm; c = 6 cm; d = 8 cm; e = 10 cm). White arrows labeled *g* and L indicate the direction of gravity and light vectors, respectively.

*2.5-Image analysis and measurement of root curvature*

At the end of each experiment run, the samples were photographed using a camera (α7 II, SONY) mounted on a photographic workstation keeping track of ECs orientation with respect to the direction of gravity and light. Images were analyzed using the package NeuronJ within the software ImageJ v1.53e (Schneider et al., 2012).

The image analysis traced a line for each root developed within the ECs providing the root length and the spatial coordinates (x, y) of the points forming the line (Figure 4). The starting point of each root was set as origin (x = 0; y = 0) considering the Y axis parallel to the gravity vector and perpendicular to the direction of light. For each root tracing, the subsequent points were detected at about 1 mm distance from each other. We then calculated the angles between the Y axis and each vector between two consecutive points of the root tracing starting from the root tip (Figure 4). The plus (+) or minus (-) sign preceding the degree of curvature indicates whether the phototropic response was positive or negative, respectively.

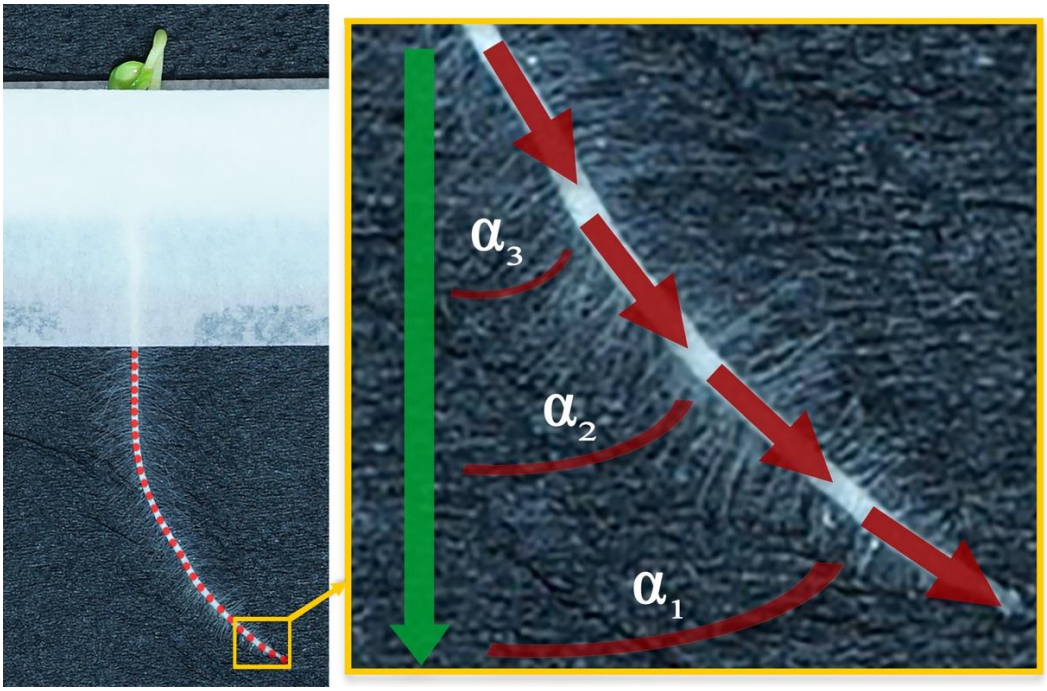


**Figure 4.** Image analysis of root growth and curvature. Red dots represent the points of the root tracing. Green arrow indicates the direction of gravity vector (Y-axis). Red arrows represent the vectors between two consecutive points of the root tracing. Angles ($\alpha_i$) were calculated between Y-axis and each vector. Illumination is from the left side of the figure. The plus (+) or minus (-) sign preceding the degree of curvature (α) indicates whether the phototropic response was positive or negative, respectively.

*2.7-Data analyses*

The influence of gravity and light, and their interaction on growth and curvature of *B. oleracea* roots was analyzed by means of generalized linear mixed model. We set the experimental replications and their interaction with treatments as random effects for the model. Pairwise comparisons were performed using Tukey's post-hoc test ($P<0.05$) to identify differences among individual treatments.

For each light treatment, a regression analysis was then used to evaluate the quantitative response of root curvature to light intensity (PFD) or gravity (*g*), setting the treatment of simulated microgravity as 0*g*. All data were processed and analyzed using Excel ver. 16 (Microsoft Corp.) and SPSS Statistics ver. 21 (IBM Corp.).

**3. Results**

Light treatments significantly affected the degree of curvature of *B. oleracea* roots grown under all gravity conditions tested in this study (Figure 5). Overall, results showed that root bending reached a photo-gravitropic equilibrium prior to the end of the 24 h treatments and the degree of curvature was steady in the last portion of the root starting from the tip (alpha 1) (Fig. 5). Unilateral blue or white light stimulated a negative phototropic response of roots. The effect after 24 h treatment was similar between blue and white light with a mean response ranging from -40° to -41° among the six gravity treatments tested in this study. Differently, at the end of the experiment, roots grown under unilateral red light showed an average degree of curvature approximately equal to 0° under hypergravity conditions (5 to 20 *g*), an angle of 13° under simulated microgravity and 5° at 1*g*. Similar to roots under red light, dark-grown roots showed a straight growth under hypergravity, and an average degree of curvature of 16° and 5° under simulated microgravity and 1*g*, respectively.

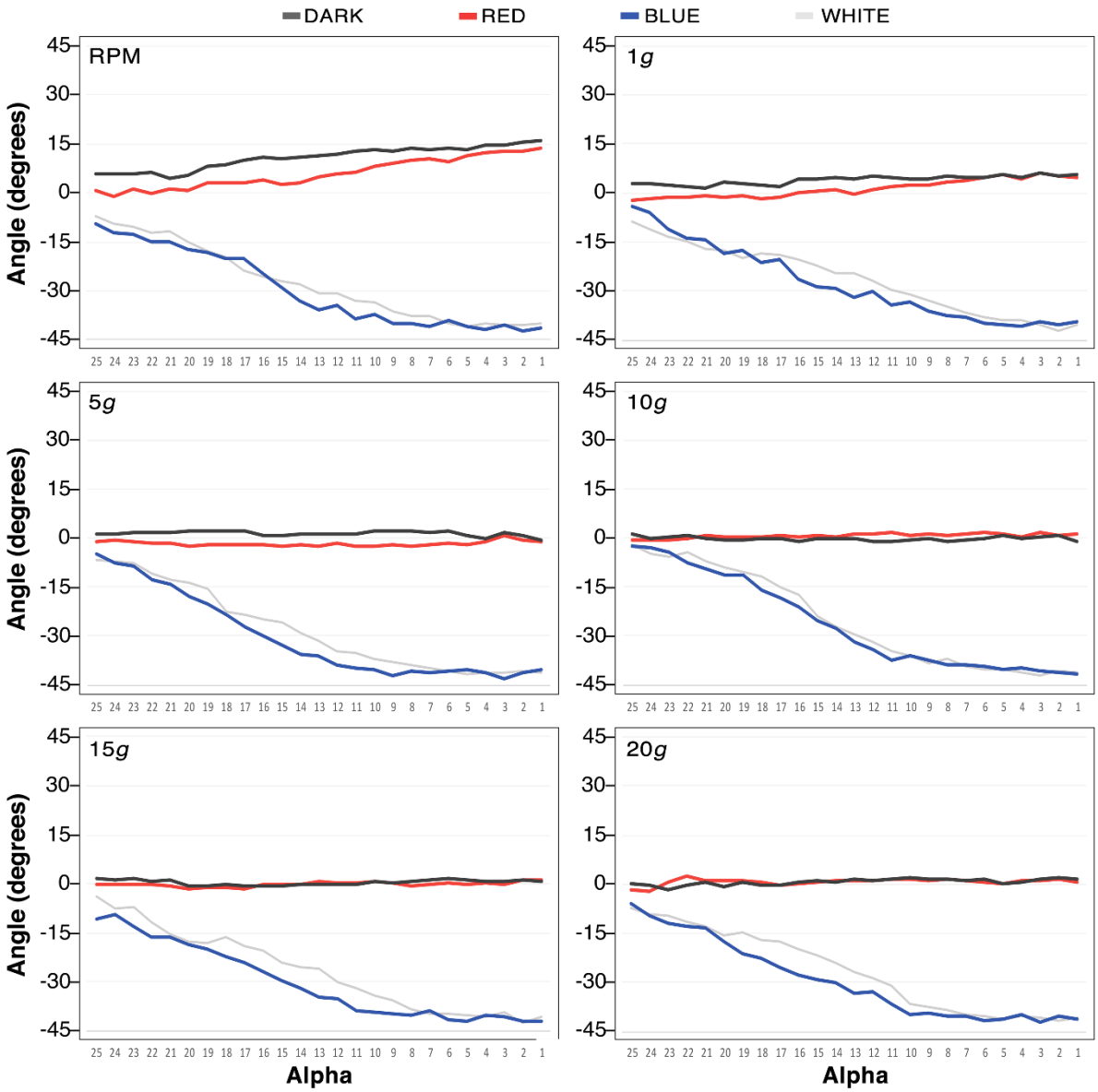


**Figure 5.** Root curvature of *Brassica oleracea* seedlings grown at different gravity levels (RPM, 1*g*, 5*g*, 10*g*, 15*g*, 20*g*) under dark conditions (dark-grey line), unilateral red light (red line), blue light (blue line), or white light (light-grey line) for 24 h. Angles were measured between the gravity vector and each vector of the root tracing starting from the root tip (alpha 1 to 25). Each data represents the mean of two replications with 25 plants. The plus (+) or minus (-) sign preceding the degree of curvature indicates whether the response was towards or away from the light source, respectively. Illumination is from the top side of the figure. Photon flux density of blue, red, and white light was ≈ 30 - 80 µmol $m^{-2}$ $s^{-1}$.

Root length was not affected by gravity level ($P$=0.83) but resulted significantly reduced under white, blue, and red light if compared to dark conditions (Figure 6). Overall, dark-grown roots were 18% longer compared to roots developed under light.

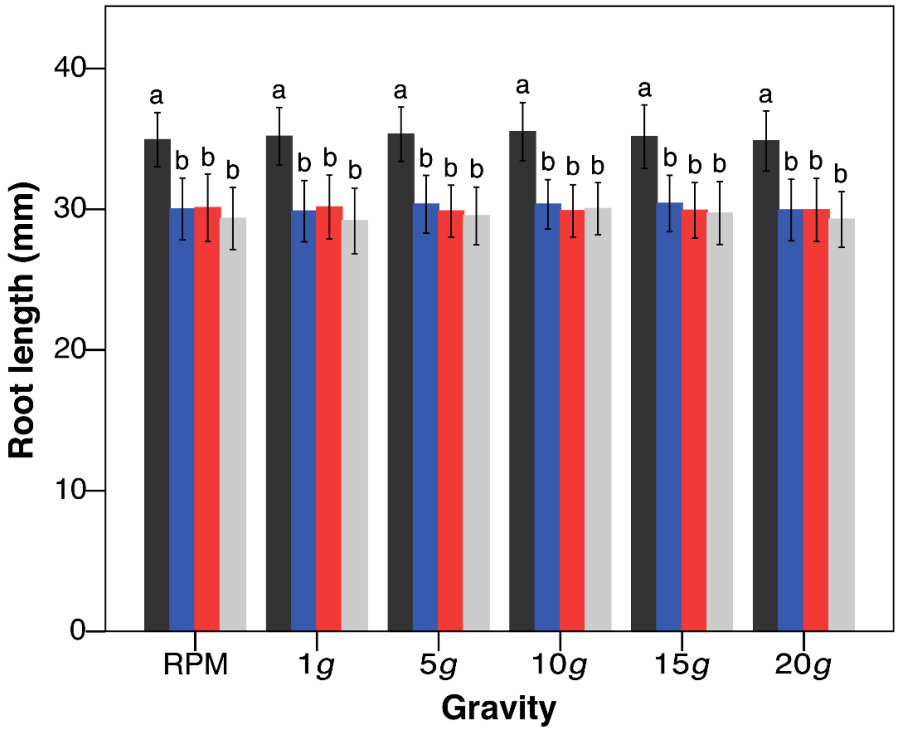


**Figure 6.** Effect of light treatments (dark conditions = dark-grey bars; red light = red bars; blue light = blue bars; white light = light-grey bars) on root length of *Brassica oleracea* seedlings grown at different gravity levels (RPM, 1*g*, 5*g*, 10*g*, 15*g*, 20*g*). Each data represents the mean and the standard deviation of two replications with 25 plants. Different letters indicate significant differences between treatments according to Tukey's multiple comparison test ($P$<0.05).

We analyzed data to highlight possible effects of increasing light intensity of white, blue, and red LEDs on root curvature. For each light treatment, the regression analysis showed no significant effect of the applied PFD on the degrees of curvature of *B. oleracea* roots and this occurred under all gravity conditions tested in this study (Figure 7).

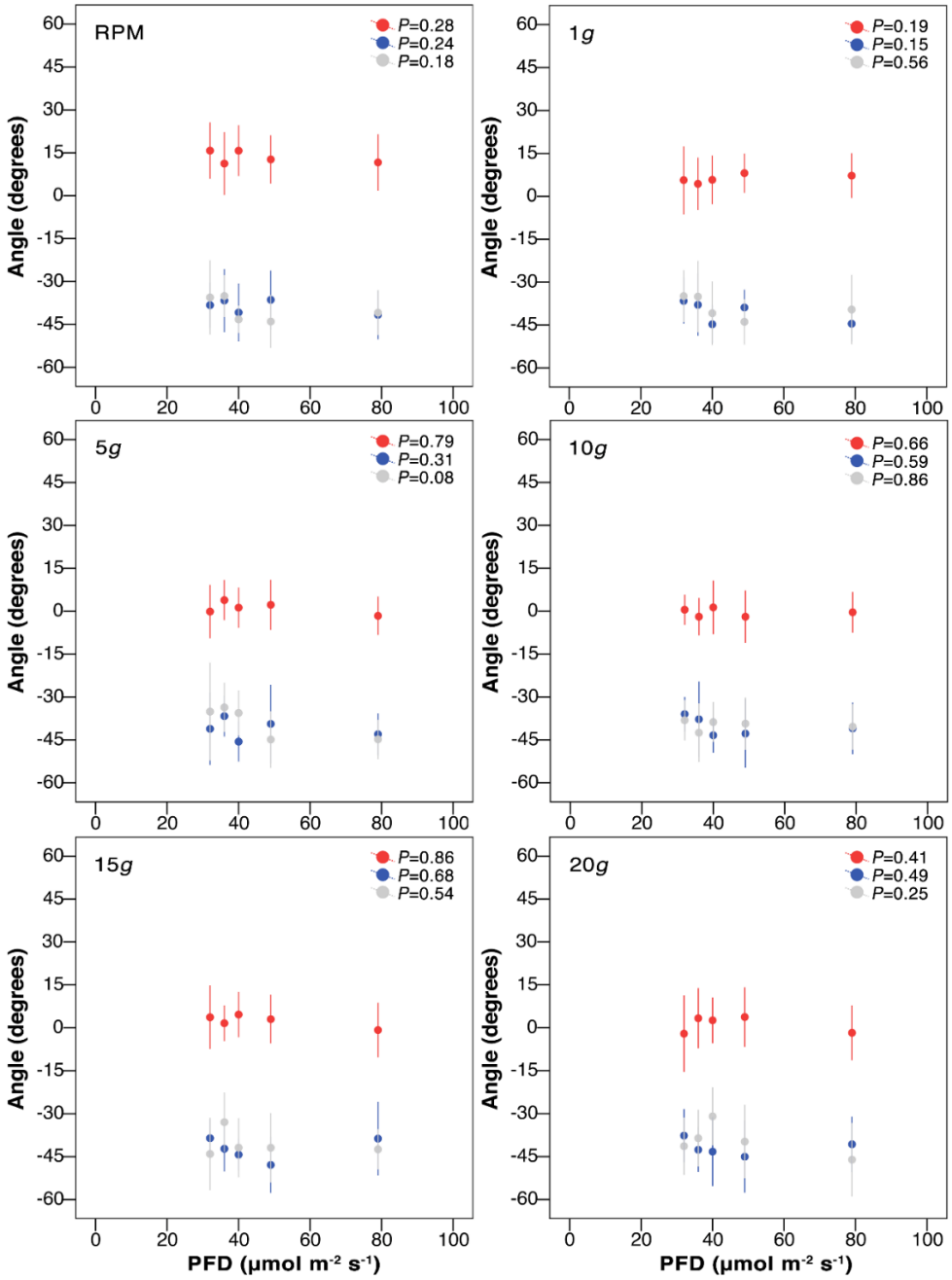


**Figure 7.** Effect of photon flux density (PFD) of blue light (blue symbols), red light (red symbols), and white light (grey symbols) on root phototropic response of *Brassica oleracea* seedlings grown at different gravity levels (RPM, 1*g*, 5*g*, 10*g*, 15*g*, 20*g*). The degree of curvature was averaged based on measurements on the last five vectors starting from the root tip (alpha 1 to 5). The plus (+) or minus (-) sign preceding the degree of curvature (α) indicates whether the phototropic response was positive or negative, respectively. Each data point shows the mean and the standard deviation of two replications with 25 plants. The *P*-values reported refer to the linear regression analysis.

The quantitative response of root curvature to the magnitude of gravity was tested in the range of simulated microgravity to 20*g*. The regression analysis showed no significant effect of gravity on root phototropic responses stimulated by white light and blue light (Figure 8) and root curvature was not affected even at 20*g* (Figure 9). Overall, the degree of curvature of *B. oleracea* roots was -41° for white- and blue-light negative phototropism, regardless of gravity. Conversely, root curvature decreased with increasing gravity under red light with an angle ranging from 13° to 0°. A similar result was also found for dark-grown roots (Figure 8).

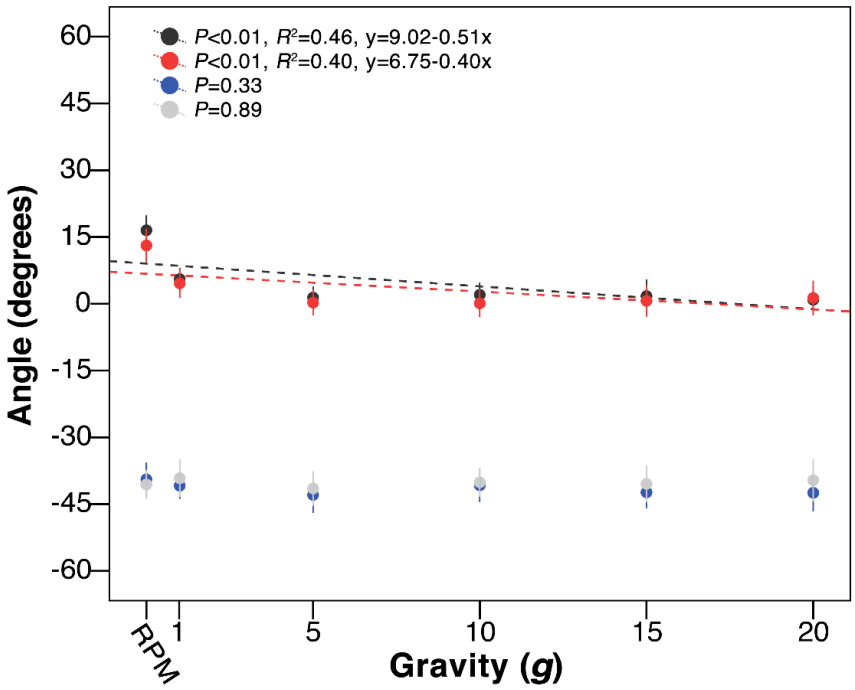


**Figure 8.** Effect of gravity levels on root phototropic response of *Brassica oleracea* seedlings grown under different light treatments (dark conditions = dark-grey symbols; red light = red symbols; blue light = blue symbols; white light = light-grey symbols). Treatment using random positioning machine (RPM) was set as 0*g*. The degree of curvature was averaged based on measurements on the last five vectors starting from the root tip (alpha 1 to 5). The plus (+) or minus (-) sign preceding the degree of curvature (α) indicates whether the phototropic response was positive or negative, respectively. Each data point shows the mean and the standard deviation of two replications with 25 plants. The *P*-values reported refer to the linear regression analysis. Dotted line represents significant linear regression.

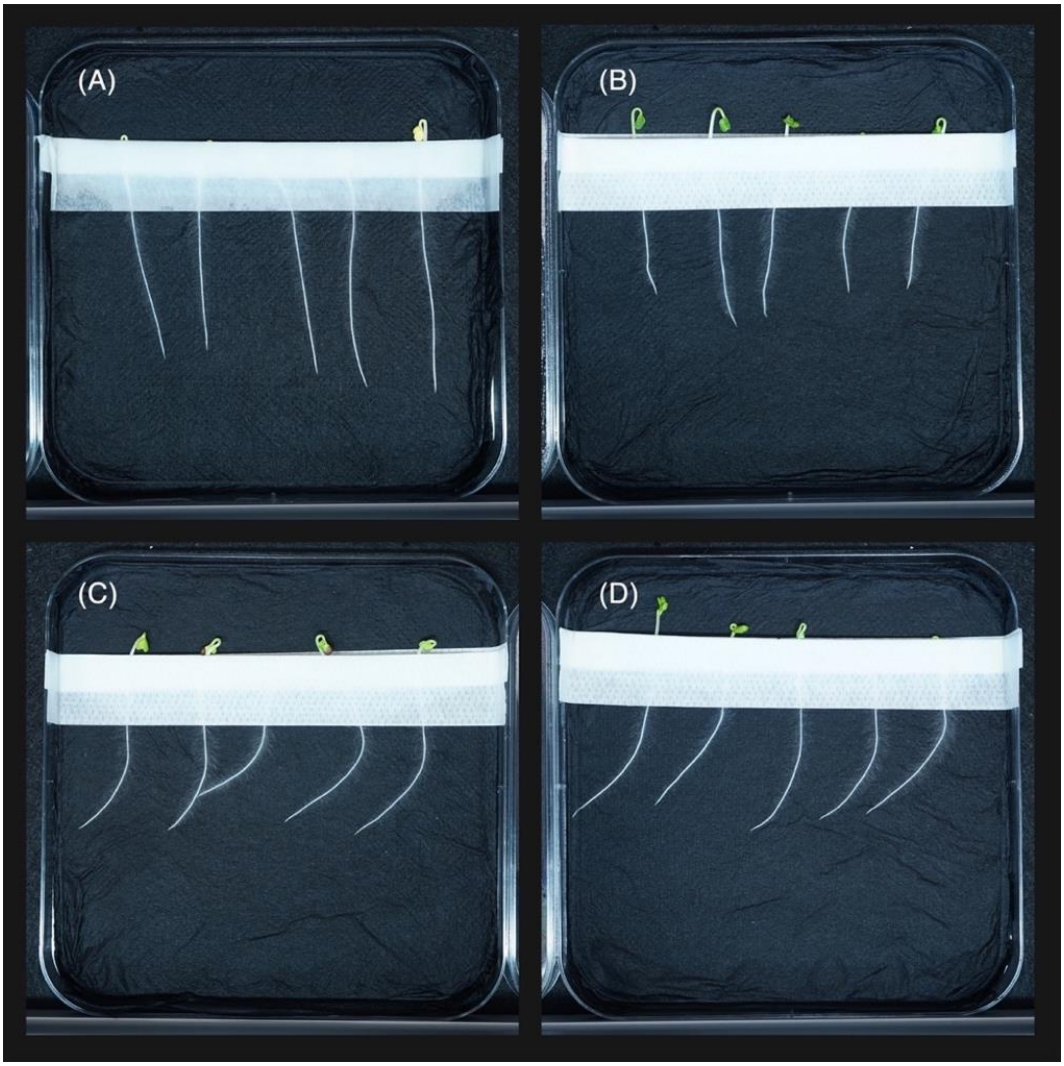


**Figure 9.** Seedlings of *Brassica oleracea* grown at 20*g* under: (A) dark conditions; (B) red light; (C) blue light; and (D) white light. Illumination is from the right side of the figure.

## 4. Discussion

It is well known that a complex mutual interaction between gravitropism and phototropism determines the form and orientation of plant roots under gravity conditions ranging from microgravity to 1*g* (Kiss et al., 2003; Kiss et al., 2012; Vandenbrink et al., 2016). To further study this interaction, we evaluated growth and curvature of *B. oleracea* roots to unilateral light treatments under different *g* levels, from simulated microgravity up to 20*g*, expanding for the first time phototropism research to hypergravity conditions.

Previous studies showed that *A. thaliana* roots exhibit negative phototropism in response to unilateral blue or white light, whereas red light can induce a positive phototropism when attenuating the effects of gravity or using mutants that are impaired in gravisensing (Okada and Shimura, 1994; Sakai et al., 2000; Ruppel et al., 2001; Kiss et al., 2012). In our study, roots of *B. oleracea* grown with unilateral white or blue light showed a negative phototropic response regardless of gravity conditions. Remarkably, the negative phototropism of roots was comparable between simulated microgravity, 1*g*, and hypergravity conditions, with an average degree of curvature of -41° measured at photo-gravitropic equilibrium. Although root curvature was similar to what was found in previous studies under 1*g* conditions, our findings do not support the vector hypothesis reported by Okada and Shimura (1994). Indeed, our data showed that hypergravity did not affect root phototropism stimulated by blue or white light, which resulted effective in orienting roots even at 20*g* (Figure 7). More specifically, Okada and Shimura (1994) reported a degree of curvature of -44° to unilateral illumination in vertically grown *A. thaliana* roots under 1 *g* conditions and using a PFD of 50 µmol $m^{-2}$ $s^{-1}$. Indeed, in their study, the light and gravity vectors were at right angles and the mean root angle was intermediate (-44°), suggesting the strength of both tropisms being equal.

Similarly, in our case, *B. oleracea* roots showed a degree of curvature of -41° stimulated by blue or white light under 1 g conditions. However, changes in the magnitude of gravity did not affect root curvature, suggesting that the gravitropic responses saturate at 1*g* or even at a lower level, and hypergravity does not reduce the effectiveness of phototropism in orienting *B. oleracea* roots. Furthermore, our results under simulated microgravity differ from previous studies on *A. thaliana* reporting either an enhancement of blue-light negative phototropism in microgravity with a significant attenuation at 0.3*g* (Kiss et al., 2012) or that a blue-light positive phototropism of roots can be revealed in microgravity (Vandenbrink et al., 2016). Nevertheless, it must be considered that the mentioned studies were performed in the near-weightless environment of the International Space Station (ISS), a condition much different from that achievable on Earth using RPM where plants constantly change their orientation with respect to the gravity vector (Kiss et al.,

2019). In this regard, further studies are needed to assess phototropic responses of *B. oleracea* in real microgravity to shed light on possible differences at species level.

The phototropic response stimulated by blue or white light was not affected by light intensity in the range of 30 to 80 µmol $m^{-2}$ $s^{-1}$ PFD. Accordingly, previous studies using *A. thaliana* found that the phototropic response increases with increasing PFD up to about 10 µmol $m^{-2}$ $s^{-1}$ and has a maximal curvature in the range of 10 to 100 µmol $m^{-2}$ $s^{-1}$ (Sakai et al., 2000; Kiss et al., 2003). Similar results have also been found in *Zea mays* by Mullen et al. (2002) who reported that blue-light phototropic response saturate at 10 µmol $m^{-2}$ $s^{-1}$. In our study, white light and blue light showed a similar effect and this can be explained considering that white LEDs provided 25% blue light which is the main driver for negative phototropism of roots. Specifically, blue-light photon flux densities of white LEDs were ≈8 µmol $m^{-2}$ $s^{-1}$ and ≈20 µmol $m^{-2}$ $s^{-1}$ at the maximum and minimum distance from the light source respectively. It is also known that other wavelengths such as green, which represented more then 50% of the emission spectrum of the white LEDs, do not affect root phototropism in a significant way (McCoshum and Kiss, 2011).

Therefore, we hypothesize that in the case of white LEDs, the root curvature of *B. oleracea* was determined by blue light component and that other wavelengths had negligible effect. Interestingly, although peak wavelength of blue (443 nm) was similar for blue and white LEDs, the intensity of blue-light photon flux was lower in white compared to blue treatment. This suggests that root curvature was not affected even at lower PFD (≈8 µmol $m^{-2}$ $s^{-1}$). In agreement with previous studies on *A. thaliana* and *Z. mays* (Sakai et al., 2000; Mullen et al., 2002; Kiss et al., 2003), our findings suggest that the response of root curvature to light intensity might be a conserved trait among numerous plant species. Nevertheless, further studies using lower PFD in the range of 0 to 10 µmol $m^{-2}$ $s^{-1}$ must determine the light-intensity threshold for root phototropism of *B. oleracea* and assess whether this response is affected by altered gravity.

As discussed above, either blue or white light stimulated a negative phototropic response of roots which was comparable among gravity treatments and light intensities. Conversely, roots under red light showed a weak curvature in simulated microgravity that was abolished when increasing the gravity level. It is known that red light can trigger a weak positive phototropism in *A. thaliana* roots which is detectable in microgravity and fractional gravity, or using mutants impaired in gravisensing (Ruppel et al., 2001; Kiss et al., 2012). However, considering that in our study a weak curvature was observed also in dark-grown roots under simulated microgravity conditions, an alternate hypothesis is that some of the root curvature might be attributed to a surface-dependent phenomenon referred as root skewing. This phenomenon involves gravity and touch stimuli resulting in a slanted angle of roots when they are growing along a nearly-vertical surface (Oliva and Dunand, 2007; Roux, 2012).

Moreover, root skewing can show significant differences in curvature and direction due to helical circumnutation of roots that can be clockwise or counterclockwise when referring to the direction of root growth (Oliva and Dunand, 2007). Similar to our study, Millar et al. (2011) reported a skew to the right of dark-grown roots of *A. thaliana* (ecotype Landsberg) during a spaceflight experiment, and this growth response was largely masked by the 1*g* conditions on Earth. Furthermore, the process called automorphogenesis has been described in seedlings germinated and grown under microgravity conditions. It consists of spontaneous curvatures of newly sprouted roots followed by straight root elongations in random directions (Hoson and Soga, 2003; Driss-Ecole et al., 2008). The molecular mechanism of automorphogenesis is still incompletely understood, as well as the relationships between automorphogenesis and skewing.

Overall, *B. oleracea* seedlings exhibited robust development under all gravity conditions with some differences in terms of growth due to the light treatments. It is known that seedlings are extremely sensitive to light quality and have evolved specific photomorphogenic responses to blue and red light which can influence both root and shoot development (Izzo et al., 2020; Izzo et al., 2021b). Still, to date, very

few studies investigated root growth response to direct illumination with spectral treatments, whereas the effect of light signals perceived by aboveground organs on root growth is more characterized (Gundel et al., 2014; Klem et al., 2019). In our study, roots were directly illuminated with white, blue, or red light from LEDs placed on one side of the EC and no significant difference was found among spectral treatments. However, dark-grown roots were longer than those grown under light, regardless of gravity conditions.

It has been shown that direct illumination of roots can shorten root length, also altering plant response to hormones or abiotic stress (Silva-Navas et al., 2015). Flavonoids, particularly quercetin, are preferentially synthesized in response to light stress and can inhibit the transport of auxin at cellular and tissue level (Brunetti et al., 2018). Indeed, the different root length between light- and dark-grown roots could be due to light-induced accumulation of flavonoids which are auxin-transport inhibitors that ultimately affect root development (Buer and Muday, 2004; Silva-Navas et al., 2016). Conversely, it has been shown that cotyledon-derived photosynthate can promote root elongation in illuminated seedlings of Arabidopsis, indicating that root development can be modulated by light through inter-organ signalling between shoots and roots (Kircher and Schopfer, 2012). However, in our experiments, the seedlings grew to an early stage of photomorphogenesis with the apical hook and cotyledons still closed which suggests that they were not fully competent to photosynthesis. Hence, it is possible that cotyledon-derived photosynthates were not significant to promote root elongation in our study, also considering the low PFD available for photosynthesis coming from the side of the EC. On the other hand, prolonged exposure to light may have inhibited root growth of illuminated compared to dark-grown roots as reported in other studies (e.g., Reboulet et al., 2010; Hopkins and Kiss, 2012; Silva-Navas et al., 2015).

It is also important to note that flavonoids have been proposed as an alternative signal gradient to auxin in mediating the differential growth response between the illuminated and non-illuminated side of the root (Silva-Navas et al., 2016). In addition, Kimura et al. (2018) concluded that the phototropic response in

Arabidopsis roots does not require asymmetric auxin distribution. Indeed, it was shown that the phototropic bending preceded the establishment of asymmetric auxin distribution, then the curvature of the root acted as a stimulus for auxin accumulation in a gravitropism-dependent manner. Similarly, Shkolnik et al. (2016) reported that auxin redistribution is not required for root hydrotropism. Taken together, these findings indicate that the Cholodny-Went theory is not applicable to all root tropisms and other unknown mechanisms should be investigated. In this regard, research in altered gravity provide opportunities to better explore the mechanisms underlying plant tropisms.

We also know that cell proliferation is affected by both microgravity and hypergravity conditions which could affect root growth and elongation (Matía et al., 2010; Manzano et al., 2012). However, illumination, either in the form of red-light photoactivation in spaceflight experiments (Valbuena et al., 2018; Villacampa et al., 2021), or the incorporation of a photoperiod regime to seedlings grown in simulated microgravity (Manzano et al., 2021), was found to attenuate or suppress the effects caused by gravitational stress at the cellular level in the root meristem. Nevertheless, no studies investigated the effect of light quality on cell proliferation under hypergravity conditions and further research is needed to deepen this subject.

*B. oleracea* has been used as model species for tropism research since the studies on plant movements by Charles Darwin in the late 1800's. Later studies in the early 1900's reported a strong negative phototropism of roots stimulated by white light in most species of Brassicaceae family, including *B. oleracea* (Schaefer, 1911; Hubert and Funke, 1937). More recently, tropism research has focused on a better understanding of the molecular pathways in model species such as *A. thaliana,* fostered by the vast database of genetic information and the availability of numerous mutants. Nevertheless, *B. oleracea* belongs to the family Brassicaceae as *A. thaliana* and can represent an ideal alternative organism due to its larger size which facilitate the sampling of target tissue (Esmon et al., 2006). Moreover, there is a substantial homology between nuclear genomes of *B. oleracea* and *A. thaliana,* and

both species exhibit time-dependent and saturable phototropic and gravitropic responses (Tatematsu et al., 2004).

In addition, *B. oleracea* is also a candidate crop for the production of microgreens as a component of life support systems in space because of its high content of phytonutrients and minerals to be integrated into the astronaut diet (Kyriacou et al., 2017). However, to date, no studies investigated growth and tropistic response of this species in microgravity, and our findings need to be verified in a true weightless environment such as found in orbiting spacecraft. As already discussed above, either blue or white light stimulated a negative phototropism in *B. oleracea* roots which was strong under all gravity conditions. From an applied science perspective (i.e., using plants as part of bioregenerative life support), the phototropic responses of *B. oleracea* can be exploited to guide root growth in a wide range of gravity conditions such as those of extraterrestrial environments.

**5. Conclusions**

Our findings provide a characterization of root gravitropism and phototropism interaction evaluating responses to different light spectra and *g* levels. We also extend phototropism research for the first time to hypergravity conditions. The use of *B. oleracea* partly confirmed results obtained in other species highlighting that some tropistic responses may be species-specific. The levels of blue or white light we applied in this study stimulated negative phototropic responses that were not affected by either simulated microgravity or hypergravity, whereas roots illuminated with red light showed a slight curvature in 1*g* and simulated microgravity but not hypergravity. Significantly, dark-grown roots showed similar responses to red light, indicating that phototropism was not involved in root curvature under red light in a significant manner and that other phenomena (including the surface-dependent touch stimuli) need to be considered. In the light of colonizing extraterrestrial environments, a thorough knowledge of photomorphogenic and phototropic responses of candidate crops such as *B. oleracea* is indispensable for plant cultivation in altered gravity. In this framework, light can represent an effective

stimulus to guide plant growth in space, and this approach can be useful in future regenerative life support systems with plants.

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# Appendix 2

# Period in the Netherlands and use of the Large Diameter Centrifuge (LDC) and the Random Positions Machines (RPM)

In addition to my Ph.D. research activities on pollen, I collaborated at those on astrobotany as team member in the ROOTROPS project funded by ESA. As part of these activities, I took part in the experiment campaign performed at the European Space Research and Technology Centre (ESA-ESTEC), Noordwijk (NL) in February 2020 for three weeks.

In this study, we tested the hypothesis that changes in light quality and magnitude of gravity could influence the net effectiveness of root gravitropism and phototropism of *Brassica oleracea* seedlings. We analyzed growth and curvature of roots under different g levels, from simulated microgravity up to 20 g, in combination with different light quality treatments. The experiment consisted of two runs, each including different gravity levels (microgravity, 1 g, 5 g, 10 g, 15 g, 20 g,) and 4 light treatments (white light, blue light, red light, and dark). The altered gravity conditions were obtained by implementing the experiments in the Large Diameter Centrifuge (LDC) and the on the Random Positions Machines (RPM) at the ESA ESTEC laboratory in collaboration with Dr. Jack van Loon. Laboratory activities

More details on ROOTROPS experiment are reported in the two published articles reported in *Appendix 1A* and *Appendix 1B* (Izzo et., al 2021; Aronne et al 2021).

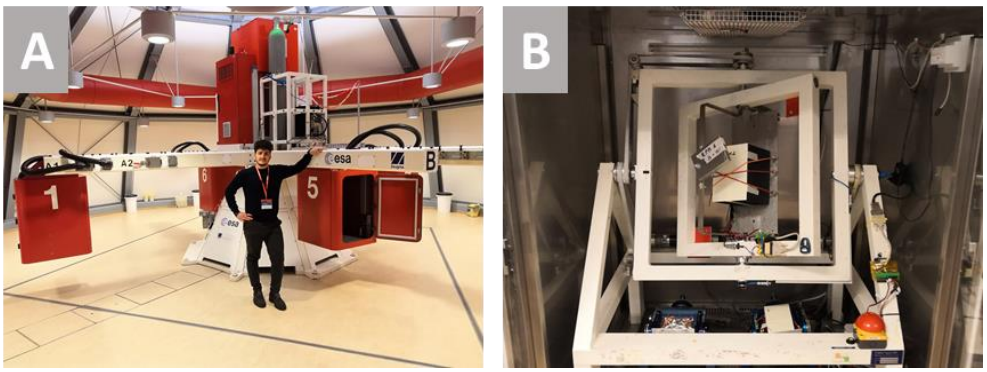


**Fig. 1.** Pictures showing the Large Diameter Centrifuge (A) and the Random Position Machine. at the European Space Research and Technology Centre (ESA-ESTEC)

# Period in Israel and use of the FiberNect

Besides the activities on pollen functionality, I was involved in the analyses on sugar nectar concentration by using a novel device named FiberNect. First description of the device and proposal to use the FiberNect for the measurements of sugar concentration of low-nectar-producing flowers has been reported in paper published on *New Phytologist* (Aronne and Malara, 2019).

The FiberNect consists of a portable fiber-optic refractometer assembled inserting all components in a 16x15x10 cm box. The device is battery powered and managed by an *ad hoc* software. The FiberNect was preliminary calibrated with known-concentration sucrose solutions and the software programmed to convert refractometer signal into equivalent sucrose concentration units (°Brix).

I have been trained to use the FiberNect and succeeded in measuring nectar sugar concentration in flowers of several species with different floral morphology, confirming that results are obtainable much more easily and quickly than with microcapillary extraction.

I showed suitability of the device in performing quick and spotted measures within different flowers in a poster presented at XVII Ecoflor Congress 2020 in Bilbao (ES).

After a further software implementation of the FiberNect, the instrument was delivered to Tel-Aviv university (IL) where I performed two month of research activities (August 2021 – September 2021) under the supervision of Professor Lilach Hadany. Specific aim of the research activities was to test the effect of pollinators sound frequencies on sugar nectar concentration in *Oenothera drummondii* flowers.

More in details, I used the FiberNect to monitor variation in sugar nectar concentration in flowers exposed to playback sounds of flying bees. The

study is still in course, but preliminary results show a consistent increase in sugar nectar concentration mostly starting within 6 minutes from pollinators sound playing (Fig. 1).

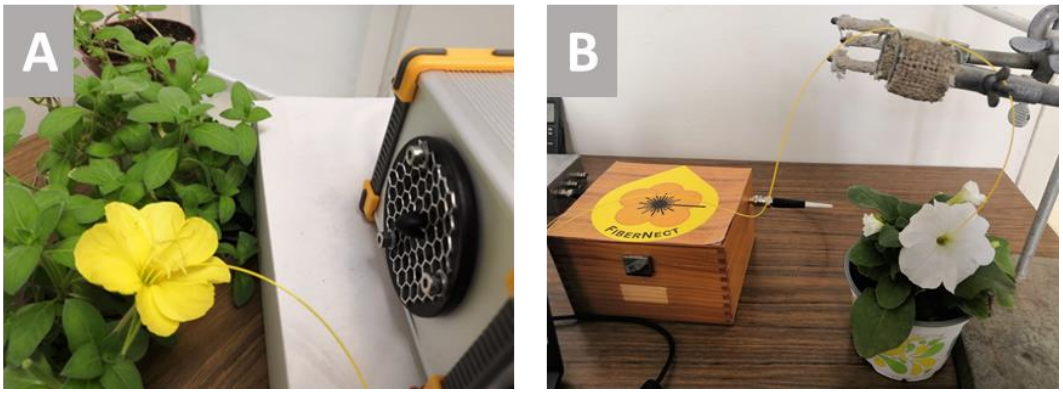


**Fig. 1.** FiberNect measuring variation in sugar nectar concentration in flowers of *Oenothera drummondii* (A). The speaker is placed closed to the flower corolla while playing bee frequencies. The yellow fiber comes from the FiberNect (B) and must be always immersed inside the nectar during the run in order to measure variations in sugar nectar concentration while the speaker is playing.

# Appendix 3

# List of publications

1. Aronne G., Iovane M., Strumia S. *Temperature and humidity affect pollen viability and may trigger distyly disruption in threatened species*. Annali di Botanica. 2021; 11: 77-82. doi: 10.13133/2239-3129/17157

2. Iovane M., Aronne G.. *High temperatures during microsporogenesis fatally shorten pollen lifespan*. Plant Reproduction, 2021; (in press). doi:10.1007/s00497-021-00425-0

3. Lisanti M.T., Mataffo A, Scognamiglio P, Teobaldelli M., Iovane M., Piombino P., Rouphael Y., Kyriacou M.C., Corrado G., and Basile B. *1-Methylcyclopropene Improves Postharvest Performances and Sensorial Attributes of Annurca-Type Apples Exposed To the Traditional Reddening in Open-Field Melaio*. Agronomy. 2021;11(6). doi:10.3390/agronomy11061056

4. Aronne G, Muthert L.W.F., Izzo L.G., Romano L.E., Iovane M., et al. *A novel device to study altered gravity and light interactions in seedling tropisms*. Life Science in Space Research. 2022, 32 (July 2021): 8-16. doi:10.1016/j.lssr.2021.09.005

5. Izzo L.G., Romano L.E., Muthert L.W.F., Iovane M., Capozzi F., et al. 2022. “Interaction of Gravitropism and Phototropism in Roots of Brassica Oleracea.” *Environmental and Experimental Botany* 193 (July 2021). https://doi.org/10.1016/j.envexpbot.2021.104700.

6. Iovane M., Cirillo A. Izzo L.G, Di Vaio C., Aronne G. *High Temperature and Humidity Affect Pollen Viability and Longevity in Olea europaea L*. Agronomy 2022, 12(1), 1; https://doi.org/10.3390/agronomy12010001

# Projects

- “ROOTROPS: tackling the roots of bending”
  Principal Investigator: Prof. Giovanna Aronne
  Partners: ESA-ESTEC Life & Physical Science (NL), Centro de Investigaciones Biológicas, Plant Cell Nucleolus, Proliferation & Microgravity (ES), e UNCG Biology Department (USA)

- ReBUS: In-Situ Resource Bio-Utilization per il supporto alla vita nello Spazio
  Partners: Agenzia Spaziale Italiana – ASI.

- COST ACTION CA18201: ConservePlants
  An integrated approach to conservation of threatened plants for the 21st Century
  Action Vice Chair: Prof. Giovanna Aronne

# Conferences

- “VI International Plant Science Conference (IPSC)”, Padova orto botanico, Italy, 4 - 7 September 2019
- XVII EcoFlor Meeting, Bizkaia Aretoa, Universidad del pais vasco, Bilbao (ES), 4 - 7 March, 2020
- “115° Congresso della Società Botanica Italiana”, Online, Italy, 9 - 11 September 2020
- MELiSSA Conference: current and future ways to closed life support system, Fully Virtual, 3 – 5 November 2020
- VII International Plant Science Conference, Online, 8 – 10 September 2021
- MedPalynoS, Online, 6 – 8 September 2021

# Oral presentations

- Iovane M., Malara P., Aronne G., FiberNect: a fiber-optic refractometer for monitoring changes in nectar sugar concentration within flowers (flash presentation), XVII EcoFlor Meeting, Bizkaia Aretoa, Universidad del pais vasco, Bilbao (ES), 4 - 7 March, 2020
- Iovane M., Aronne G., High temperatures during microsporogenesis affect functionality of pollen and timing of developmental stages, VII International Plant Science Conference (IPSC), Online, Italy, 9 - 11 September 2020
- Iovane M., Izzo L. G., Aronne G., High temperatures affect pollen fertility more than altered gravity: bottlenecks in the reproductive cycle of Micro-Tom, MELiSSA Conference: current and future ways to closed life support system, Fully Virtual, 3 – 5 November 2020
- Iovane M., Aronne G., Heat treatment during microsporogenesis affects thermo-tolerance and ontogenesis of tomato pollen, MedPalynoS, Online, 6 – 8 September 2021

# Posters

- Iovane M., Izzo L.G., Aronne G. High temperatures affect pollen viability of Micro-Tom: a model crop for seed-to-seed cycle in space. VI International Plant Science Conference (IPSC), Padova orto botanico, Italy, 4 - 7 September 2019
- Iovane M., Aronne G., High temperatures during microsporogenesis affect functionality of pollen and timing of developmental stages, 115° Congresso della Società Botanica Italiana, Online, Italy, 9 - 11 September 2020
- Iovane M., Malara P., Aronne G., FiberNect: a fiber-optic refractometer for monitoring changes in nectar sugar concentration within flowers, XVII EcoFlor Meeting, Bizkaia Aretoa, Universidad del pais vasco, Bilbao (ES), 4 - 7 March, 2020
- Iovane M., Izzo L. G., Cirillo A., Romano L. E., Bruno A., Di Vaio C., Aronne G., Pollen from 13 cultivars of Prunus armeniaca L. differently reacts to temperatures in a climate change scenario. VII International Plant Science Conference, Online, 8 – 10 September 2021

# Attended courses

1. *"How to prepare a research paper and present experimental data"*, Department of Agricultural Sciences, University of Naples Federico II, November – December 2019
2. *"Applied Statistics for Scientific Research",* Department of Agricultural Sciences, University of Naples Federico II, October - December 2020
3. *"Analysis and management of agronomic, food and environmental data in matlab"*, Department of Agricultural Sciences, University of Naples Federico II, November 2019 – April 2020
4. *"Data science & computational analysis for metagenomics",* Department of Agricultural Sciences, University of Naples Federico II, January – May 2019
5. *"WORKSHOP Effective scientific presentations"* Bizkaia Aretoa, Universidad del pais vasco, Bilbao (ES), March 9th, 2020

# Attended seminars

1. *La bioinformatica come disciplina trasversale a sostegno delle scienze agrarie",* Department of Agricultural Sciences, University of Naples Federico II, May 13th, 2019
2. *"La tolleranza agli stress, la qualità e la fisiologia vegetale*", Department of Agricultural Sciences, University of Naples Federico II, October 10th, 2018
3. *"Crop adaptation to climate change",* Department of Agricultural Sciences, University of Naples Federico II, November 28th, 2018
4. *"Data science per lo studio di ecosistemi complessi",* Department of Agricultural Sciences, University of Naples Federico II, June 26th, 2019
5. *"Inside-out. The inhibitory effect of self-DNA",* Department of Agricultural Sciences, University of Naples Federico, February 24th, 2021
6. *"Gestione della fertilità degli agroecosistemi ispirata dal dialogo tra pianta, suolo e batteri"* Department of Agricultural Sciences, University of Naples Federico, December 15th ,2021