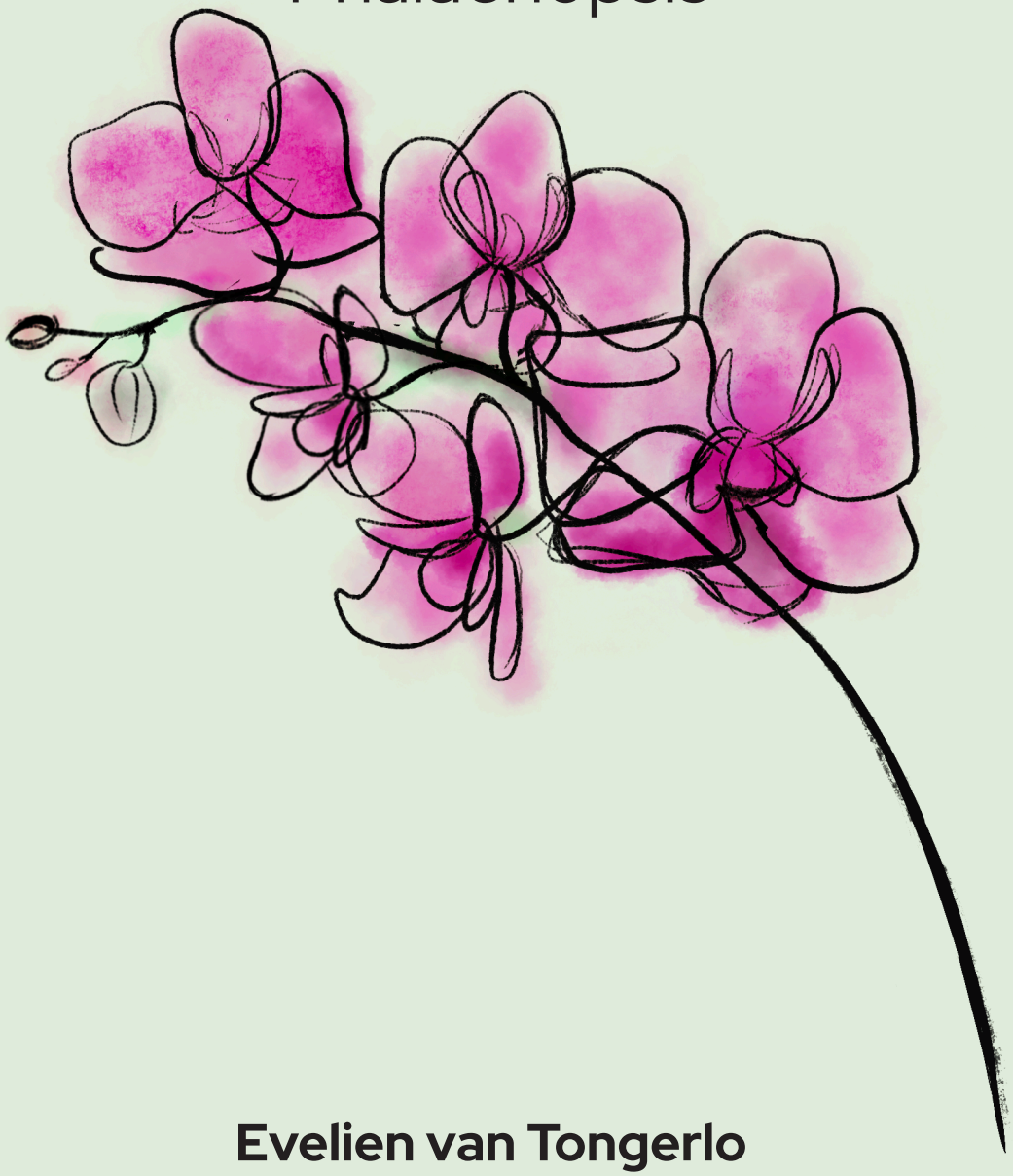
From diel photosynthesis to crop growth in the crassulacean acid metabolism (CAM) orchid Phalaenopsis



Evelien van Tongerlo

Propositions

1. Diel CO_2 uptake expressed per plant during vegetative growth can be used as a proxy for flowering quality in *Phalaenopsis*.
   (this thesis)

2. Crassulacean acid metabolism (CAM) crops are an essential part of climate-resilient agriculture.
   (this thesis)

3. Innovation can only occur when there is interaction between science, policy, society, and practice.

4. There is a lack of research on the critical success factors that affect the current use and impact of artificial intelligence (AI).

5. AI algorithms would be more useful if they explained the processes behind their decisions.

6. Binary thinking hampers social and intellectual progress.

7. The continuous focus on happiness is causing a lot of misery.

Propositions belonging to the thesis, entitled

From diel photosynthesis to crop growth in the crassulacean acid metabolism (CAM) orchid Phalaenopsis

Evelien van Tongerlo
Wageningen, 2 July 2021

From diel photosynthesis to crop growth in the crassulacean acid metabolism (CAM) orchid Phalaenopsis

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From diel photosynthesis to crop growth in the crassulacean acid metabolism (CAM) orchid Phalaenopsis

Evelien van Tongerlo

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Contents

List of abbreviations

C_a,C_i	ambient, internal CO_2 concentration (ppm)
CAM	crassulacean acid metabolism
CCM	carbon concentrating mechanism
DLI	daily light integral (mol photons m^{-2} day^{-1})
DW	dry weight (g)
ETR	electron transport rate
Glc eq	glucose equivalents (mmol m^{-2})
g_m	mesophyll conductance (mol H_2O m^{-2} s^{-1})
g_s	stomatal conductance (mol H_2O m^{-2} s^{-1})
GxE	genotype x environment
LMA	leaf mass area (kg m^{-2})
NAD(P)-ME	NAD(P)-dependent malic enzyme
NPQ	non-photochemical quenching
OAA	oxaloacetic acid
PC	principal component
PCA	principal component analysis
PEP	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxylase
PEPCK	phosphoenolpyruvate carboxykinase
PPCK	phosphoenolpyruvate carboxylase kinase
PPFD	photosynthetic flux density (µmol m^{-2} s^{-1})
PSII	photosystem II
PSS	phytochrome photostationary state
R:FR	red:far red
RGR	relative growth rate (g d^{-1})
Rubisco	ribulose-1,5-bisphosphate carboxylase-oxygenase
SLA	specific leaf area (m^2 kg^{-1})
SPAC	soil-plant atmosphere continuum
VPD	vapour-pressure deficit (kPa)

WUE	water-use efficiency (mol CO_2 assimilated per mol H_2O transpired)
ΦPSII	photosynthetic operating efficiency

Chapter 1
General introduction

1. Crassulacean acid metabolism

Crassulacean acid metabolism (CAM) is a specialized photosynthetic adaption that occurs in approximately 6% of all vascular plants (Winter and Smith, 1996). It was first discovered in the plant species formerly known as *Bryophyllum calycinum* (Salisb.) (Ranson and Thomas, 1960), now named *Kalanchoe pinnata* (Lam.). These plants were found to accumulate substantial amounts of acid during the night, which decreased again during the day (deacidification). This happens because CAM plants temporally separate the uptake of CO_2 into the mesophyll from fixation by ribulose-1,5-bisphophatase carboxylase oxygenase (Rubisco). The nocturnally fixed CO_2 is stored in the vacuole, in the form of a carboxylic acid. CAM crops, such as *Agave* sp., *Aloe* sp., *Opuntia* sp., *Ananas comosus* (L.) Merr. (pineapple), and several orchids such as *Vanilla* Mill. and *Phalaenopsis* sp. are grown for food, as ornamentals or as biomass for fuel (Davis et al., 2019). CAM is usually not considered an advantageous characteristic for crop production, although more recently, an increasing number of studies acknowledge the potential of CAM in agriculture (DePaoli et al., 2014, Yang et al., 2015, Owen et al., 2016, Davis et al., 2019). CAM plants have high water-use efficiency (WUE) and can grow on arid, semi-arid lands where agriculture is not, or is no longer, possible or feasible (Yang et al., 2015). Instead of the detrimental continuation of land conversion to cropland, resulting in e.g. increased soil erosion (Borrelli et al., 2017), CAM offers an opportunity to reclaim marginal lands. One way to improve future food and energy security in a rapidly changing world where the effects of climate change become more and more noticeable, is by increasing our understanding of CAM plants.

1.1. CAM-phases in a diel cycle

Unlike C_3 and C_4 plants, CAM plants keep their stomata closed during the day, and CO_2 uptake occurs mainly in the night. This gives CAM plants a competitive advantage in areas where water or CO_2 is limiting (Cushman et al., 2000). The regulation the temporally separated distinct processes, is mainly under circadian control (Hartwell, 2006). However, the rigidity imposed by the circadian clock is toned down by the complex interplay of metabolite control and environmental cues (Dodd et al., 2002). To describe the diel cycle of CO_2 uptake and stomatal conductance in CAM plants, Osmond (1978) proposed a framework that consists of four

phases. This framework is still being used to this day (Figure 1.1), and will be described in more detail below.

1.1.1. Phase I

During the night when evapotranspiration rates are low, stomata are open (phase I), CO_2 is taken up, and fixed by phosphoenolpyruvate carboxylase (PEPC)(Figure 1.2). The source of CO_2 can either be atmospheric, or from respiration. CO_2 (in the form of HCO_3^-) is fixed to the acceptor phosphoenolpyruvate (PEP), a 3-C substrate that is recovered from stored carbohydrates (starch and/or soluble sugars) via glycolytic breakdown, and oxaloacetate (OAA) is formed. Subsequently, OAA is pumped into the vacuole of a mesophyll cell as a C_4 carboxylic acid; usually malate, sometimes citrate or isocitrate (Lüttge, 1990). This is done by creating a positive transmembrane electrical potential across the tonoplast (either via a H^+-ATPase pump or inorganic pyrophosphatase (PP_iase)(Smith et al., 1996, Holtum et al., 2005). While PEPC protein abundance does not change over a diel cycle, its activity is regulated via phosphorylation by phosphoenolpyruvate carboxylase kinase (PPCK), which activates PEPC and renders it less sensitive to inhibition by malate (Borland and Taybi, 2004). In its phosphorylated state, PEPC has a six times higher affinity for CO_2 than Rubisco (Lüttge, 2001). Therefore, regulation of carboxylases is essential for CAM to function.

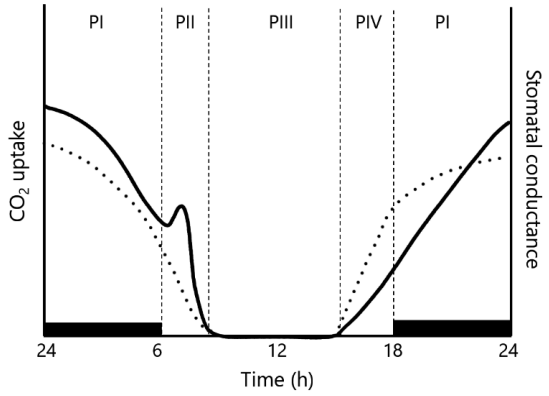

Figure 1.1 Schematic representation of a diel pattern of CO_2 uptake (solid line) and stomatal conductance (dotted line) of a CAM plant, showing the four phases as defined by Osmond (1978). Stomatal conductance pattern adapted from Males & Griffiths (2017).

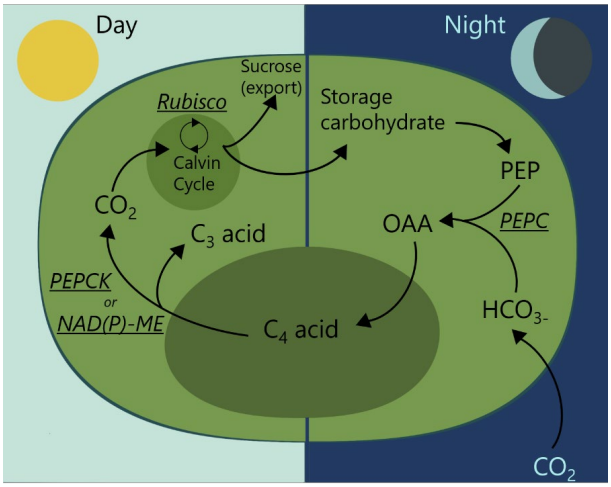

Figure 1.2 Schematic outline of the most important processes and fluxes in plants with crassulacean acid metabolism (CAM). Enzymes in underlined italics. Abbreviations: OAA, oxaloacetic acid; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; NAD(P)-ME, NAD(P) dependent-malic enzyme; PEPCK, phosphoenolpyruvate carboxykinase; Rubisco, ribulose-1,5-bisphophatase carboxylase oxygenase.

1.1.2. Phase II

Phase II takes place at dawn, when stomata are still open. During phase II, the plant switches from carboxylation by PEPC to carboxylation by Rubisco, as light intensity increases. Meanwhile, PEPC activity decreases as it is dephosphorylated, whereas Rubisco activity increases (Maxwell et al., 1999). As both carboxylases are active during this phase, a burst of CO_2 uptake can occur (Winter and Tenhunen, 1982). When the tonoplast switches from influx to efflux, carboxylic acid is passively released from the vacuole, and decarboxylated, via NAD(P)-ME and/or PEPCK (Christopher and Holtum, 1996). This generates a high internal pressure of CO_2, which causes stomata to close and marks the start of phase III.

1.1.3. Phase III

During this phase, CO_2 that is decarboxylated continues to diffuse into the chloroplast where it is fixed by Rubisco (Figure 1.2). High pCO_2 suppresses photorespiration throughout phase III, when stomata are closed (Cushman, 2001). However, photorespiration in CAM plants is not absent, and they possess (and require) a completely functioning photorespiratory pathway (Busch, 2020). Therefore, the current consensus is that CAM, like C_4, should be considered a carbon concentrating mechanism (CCM). As decarboxylation of nocturnally stored C_4-acids

commences, regeneration of storage carbohydrate from the remaining 3-C compound (pyruvate or PEP) occurs via gluconeogenesis (Osmond et al., 1996). This ensures that sufficient substrate is available for the following night. Maintaining the daily carbon turnover required for functioning of the CAM cycle can take up to 20% of the plant's carbon budget (Borland and Dodd, 2002).

1.1.4. Phase IV

When all CO_2 that was stored is depleted, pCO_2 drops, which is the main reason for stomata to re-open. Net CO_2 uptake marks the start of phase IV. Throughout phase III, Rubisco activity continues to increase, and reaches maximum levels early in phase IV. During this phase, CO_2 uptake occurs in the light. It is believed that this occurs mainly via Rubisco (Maxwell et al., 1999). Therefore, phase IV most closely resembles C_3 fixation. Due to low internal conductance in leaves of CAM plants (Maxwell et al., 1997), direct CO_2 fixation via Rubisco in phase II and IV might result in significant photorespiration (Borland et al., 2000). During phase IV, phosphorylation of PEPC is upregulated and fixation of CO_2 into carboxylic acid recommences, until the night marks the start of another phase I. Transitional phases II and IV respond very sensitively to environmental conditions. Occurrence and duration strongly depend on temperature, light level and water status of the plant (Lüttge, 2004). If conditions are unfavourable, stomata can remain closed during phase II and IV to save water. Therefore, these phases are not always part of the diel CAM cycle (Winter, 2019).

1.2. Energetic costs of CAM

The temporal separation of carboxylases comes with extra energetic costs, compared to C_3 fixation of CO_2. Additional ATP is needed for regeneration of the 3-C compound via gluconeogenesis during the day, and for pumping C_4 acid into the vacuole at night (Borland et al., 2009). Additional reducing power is primarily needed during the night to synthesize malate from OAA (Shameer et al., 2018). Interestingly, computational analysis indicates that the final impact on productivity appears trivial (Nobel, 1991, Shameer et al., 2018), which is due to two major adaptions that occur in CAM plants. Firstly, the additional cost of running CAM is countered by the suppression of photorespiration in phase III (Cushman, 2001). Photorespiration can increase the cost of CO_2 fixation in C_3 plants by 25% (Nobel 1991), and

because this is mostly suppressed in CAM plants, the cost of CO_2 fixation can be reduced. Secondly, the main route of starch degradation in CAM plants seems to differ from what is known from C_3 (Borland et al., 2016). Shameer et al. (2018) calculated that the nocturnal breakdown of starch was up to 8.7 times higher in CAM plants, compared to C_3. However, while C_3 plants make use of the hydrolytic pathway for starch breakdown, CAM plants mainly use the phosphorolytic starch degradation route to provide substrate for PEP. This pathway is associated with lower energetic costs, as it can provide ATP (Holtum et al., 2005, Shameer et al., 2018). The exact stoichiometry of ATP and NADPH in CAM plants depends on the type of tonoplast pump, storage carbohydrate, decarboxylation enzyme, and subsequent carbohydrates produced in the light.

1.3. Phenotypic diversity in CAM

CAM plants grow in a harsh and highly disruptive environment, which requires high photosynthetic plasticity, allowing them to rapidly adapt to changes in the environment (Dodd et al., 2002). The contribution of CO_2 fixation via CAM relative to C_3 can vary from <1% to 100% of total carbon gain (Winter, 2019). This raises questions on how to define a CAM plant. Winter et al. (2015) state that only plants that take up a substantial part of CO_2 throughout the majority of their lifecycle via the CAM pathway, deserve the qualification 'CAM species'. Despite this proposed definition, it is difficult to capture CAM as a trait, as both ontogenetic and environmental factors affect CO_2 fixation via CAM (Figure 1.3). In obligate (or consecutive) CAM species, CAM will develop in mature photosynthetic tissues, regardless of the environment (Winter, 2019). Young tissue of obligate CAM plants expresses C_3 photosynthesis, which gradually shifts to fixation via CAM, for example in *Kalanchoe pinnata* (Winter and Holtum, 2014). Obligate CAM plants still show plasticity in how different phases contribute to the diel cycle. Facultative CAM plants engage in CAM only under stress, such as drought or salt stress (e.g. Haider et al., 2012). The induction of CAM is reversible, and these plants can revert to C_3 (see Winter (2019) and references therein) or even C_4 photosynthesis (e.g. *Portulaca sp.*; Ferrari et al., 2020). Both obligate and facultative CAM species can engage a form of CAM idling. During CAM idling, plants keep their stomata almost completely closed and the CAM cycle is fed internally, by recycling respiratory CO_2. This is considered the strongest form of CAM, and can occur in response to severe water stress (Lüttge, 2004). CAM cycling can be considered the

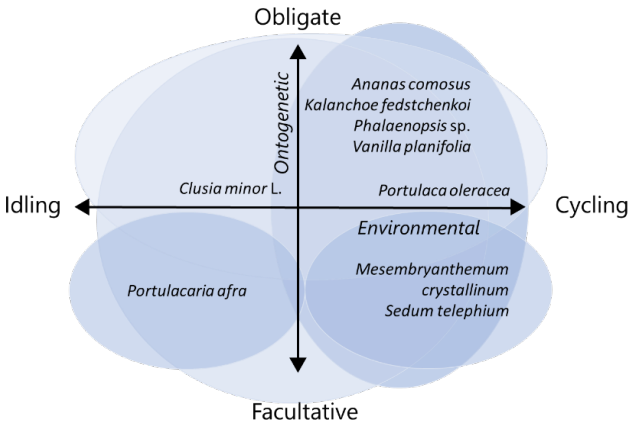

Figure 1.3 Spectrum of phenotypic variation in plants with crassulacean acid metabolism (CAM), ontogenetically ranging from facultative (also: C_3/C_4-CAM intermediate) to obligate, and in response to the environment from idling (weak) to cycling (strong). Some examples are placed within the spectrum, but their position can shift depending on ontogeny and environment. See for *Ananas comosus* (Yang et al., 2015), *Portulaca oleracea* (Ferrari et al., 2020), *Vanilla planifolia* (Gehrig et al., 1998), *Clusia minor L.* (Lüttge, 2006), *Phalaenopsis* (Chen et al., 2008, Ceusters et al., 2019). For all other species and a more complete overview , see Sayed (2001).

opposite of this, as it is the weakest form of CAM. Plants that engage in CAM cycling keep their stomata closed during the night, but respiratory CO_2 is used to feed the CAM cycle and some nocturnal accumulation of acid occurs (Cushman, 2001). However, during the day stomata are opened normally, as in C_3 plants. The wide spectrum of plants that engage in CAM, their habitats, and their metabolic flexibility makes it difficult to generalize statements on this photosynthetic adaption.

Nevertheless, CAM plants do share some common anatomical traits that contribute to water saving strategies and CAM functioning (Borland et al., 2018). Morphological features such as thick leaves, succulence, large and tightly packed mesophyll cells, causing low intercellular air space and reduced surface of mesophyll exposed to intercellular air space, are associated with CAM (Nelson and Sage, 2008). According to Winter (2019), strong CAM is associated with higher succulence. However, this correlation seems to hold only to a certain point, beyond which CAM functioning is not further enhanced (Nelson and Sage, 2008). Furthermore, the link between leaf anatomical traits and CAM mode should be made with caution, as this might hold within one family, but not when species are pooled (Herrera, 2020).

1.4. Considerations when bio-engineering CAM into C_3

Recently, Edwards (2019) postulated that when CAM plants evolved from C_3 plants, biochemistry evolved first, which was then followed by changes in anatomy. CAM has evolved independently several times in diverse lineages, and in all areas of the world. Theoretically, all enzymes and relevant processes that are needed for CAM are also present in C_3 (Edwards, 2019). If CAM could be engineered into C_3 plants, important food crops such as wheat could benefit from increased WUE. Yang et al. (2017) have studied the genomic data of *Kalanchoe* and found that convergent evolution of CAM occurred between eudicots (like *Kalanchoe*) and monocots (like pineapple and *Phalaenopsis* orchid). Their analysis pinpoints several candidate-genes that could help with engineering CAM into C_3 crops. This could make C_3 crops more resilient to hotter and drier conditions, at a limited productivity penalty (Töpfer et al., 2020). Until recently, little attention was given to the functioning of CAM crops in temperate regions where these C_3 crops grow. This is also true for production of crops in a horticultural setting, like for *Phalaenopsis, Kalanchoe*, and many other CAM ornamentals, e.g. several species from the *Bromeliaceae*. Theoretically, at least 25% of water can be saved when plants have a CAM(like)-mode (Töpfer et al., 2020). The temperate regions in which C_3 crops grow might benefit more from CAM-C_3 intermediate crops that can engage either photosynthetic type when needed, than bio-engineering full CAM into C_3 plants. If environmental conditions are favourable, C_3 can be used for rapid growth, whereas drought periods are covered by switching to CAM (Winter, 2019).

2. Orchids[1]

Considered one of the largest families of angiosperms, the *Orchidaceae* comprises over 29 000 species and can be found in all inhabited continents (Swarts and Dixon, 2009, Hinsley et al., 2018), although they are most common in the tropics. Only a small number of genera are commercially cultivated, all of which belong to subtribes and genera that show CAM in their lineage. However, CAM cannot generally be assigned to a whole genus, since CAM may occur in some species but not in others, and it is unclear how abundant CAM is in orchids (Arditti,

[1]A revised version of the paragraph "2. Orchids" has been published as part of Davis et al., 2019; Journal of Experimental Botany **70**(22): 6521-6537

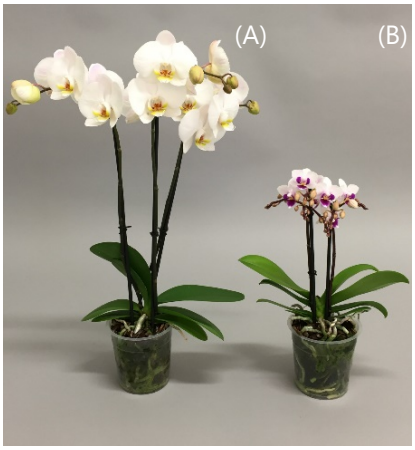

Figure 1.4 Flowering *Phalaenopsis* orchids. Cultivars can have fewer, but larger flowers and buds (A), or are smaller and have a higher number of small flowers (B).

1992; Silvera et al., 2009; Winter, 2019). *Vanilla* is the only genus that is commercially grown for its edible fruit with relevant flavour and aroma compounds (De La Cruz Medina et al., 2009). There are several other uses for orchids, such as production of flour made from orchid tubers called *salep* in the eastern Mediterranean and Middle east, and *chikanda* cake in south-eastern Africa. Various orchid species are used in traditional Chinese medicine and health supplements (Fay, 2018). The orchids used for these purposes are harvested only in the wild. Although their use might be minor and limited to specific regions, there are growing concerns that collection and trade of these wild orchids will result in scarcity or even extinction (Liu et al., 2014, Fay, 2018).

The most common use of orchids is as ornamentals. The most important genera for cut orchids are *Cymbidium, Oncidium, and Phalaenopsis*, although the latter is mostly sold as a potted plant. Interestingly, orchids were considered a minor crop by the USDA until 1997, and no product information was collected (Lopez and Runkle, 2005), but in recent times, this has changed. In the USA, 21 million potted *Phalaenopsis* plants were sold in 2012 (USDA-NASS, 2016), representing a wholesale value of 177 million US dollars, and accounting for 19% of the potted plant market. In Europe, 2017 figures from the Dutch flower auction (Royal Flora Holland, 2018) indicate that 135 million *Phalaenopsis* plants were sold, representing a turnover of 494 million euros (32% of total potted plants revenue). Reliable propagation techniques and control of the flowering process allow for high quality plants with large, showy flowers (Figure 1.4) (Lopez and Runkle, 2005, Runkle, 2019). *Phalaenopsis* engages CAM photosynthesis.

Therefore, *Phalaenopsis* offers an opportunity to further study the functioning of CAM, in a crop that is considered an important commodity in the horticultural sector (Runkle, 2019).

2.1. A note on nomenclature

About 70 different species belong to the genus *Phalaenopsis*. However, the popularity of *Phalaenopsis* has led to the development of many artificial hybrids (Griesbach, 2002). Hybrids combine the qualities of different *Phalaenopsis* species or varieties. Hybridization results in plants that no longer belong to one or the other species. In this thesis, I have worked only with *Phalaenopsis* hybrids. The name *Phalaenopsis* therefore refers to hybrids of *Phalaenopsis* species, not to the complete genus or one species, unless otherwise specified.

2.2. Phalaenopsis

Phalaenopsis orchids are epiphytes, that can be mainly found in tropical rainforests in south-east Asia. These ever-green forests are characterized by relatively humid, warm and relatively shaded conditions (Christenson, 2001). Epiphytes have no direct access to soil, and have evolved novel strategies in order to deal with intermittent rainfall and limited nutrient availability, such as the water-storage-tanks in *Guzmania sp.*, water-capturing strategies of *Tillandsia* sp., as well as the development of CAM in *Phalaenopsis* (Niechayev et al., 2019, Gilman and Edwards, 2020). *Phalaenopsis* plants have green, succulent leaves, that can contain a certain amount of anthocyanins that cause leaves to turn red or to show greyish flecks.

Figure 1.5 Per leaf, two undifferentiated axillary buds are formed (A). The larger bud usually develops into a flower spike. When damaged, the lower, still dormant bud, can develop into a flower spike (B).

Phalaenopsis leaves are differentiated alternately on opposite sides of the stem, a phyllotaxis that is unfavourable for light interception. *Phalaenopsis* plants show large variation in leaf size and morphology (e.g. leaf angles). Between leaves, aerial roots are visible, that are enveloped by a velamen. The velamen is a spongy epidermis that highly efficient in absorbing and retaining atmospheric water and nutrients (Zotz, 2016). Roots also contain chloroplasts, and can engage CAM-photosynthesis as well (Motomura et al., 2008). Per leaf, two axillary, undifferentiated, dormant buds are present (Figure 1.5A)(Blanchard and Runkle, 2006). If a flower spike is formed, it is the top axillary bud that develops. The second, smaller bud remains dormant, unless the top bud is damaged or removed (Figure 1.5B). In practice, only one flower spike develops per leaf, which penetrates the leaf sheath on the lower side of the leaf. Usually, the first flower spike arises from an axillary bud in the leaf axil of the fourth leaf counted from the top (Sakanishi et al., 1980). Each flower spike is composed of several extending internodes, allowing it to rise above the plant. Every internode has its own axillary bud, that can develop into a side shoot (branching). The flower spike can be a panicle or raceme. Differentiation and development of flower buds starts when the flower spike is 5-10 cm long, but only when environmental factors are still favourable.

2.2.1. Commercial cultivation of Phalaenopsis

To ensure year-round production, commercial cultivation of *Phalaenopsis* occurs in greenhouses, but their growth is slow and costly. Depending on the cultivar, it can take over two years to produce a marketable product after propagation in the lab (Paradiso et al., 2012). The vegetative growth phase takes approximately 50-70 weeks and is needed to ensure sufficient plant size. In this phase, leaf initiation is the key developmental process, as at least five new leaves have to be developed (Paradiso et al., 2012). Plant size, as well as number of leaves are thought to positively correlate with flowering potential (Sakanishi et al., 1980, Runkle, 2019). Vegetative growth is followed by a forcing (or: cooling) phase, with lower temperatures (<25°C, but in practice around 20°C) that are needed to induce flowering. This phase takes 6-9 weeks (Paradiso et al., 2012). Flower development and outgrowth takes another 8-10 weeks, before the plant is marketable; usually after anthesis of the first two flowers. When temperatures during flower development are too high, the flower spike can form a vegetative shoot instead of developing flowers (Figure 1.6)(Lopez and Runkle, 2005). This suggests that

Figure 1.6 Vegetative shoot formed from axillary bud (also known as 'keiki'), instead of a flower spike.

two key moments determine if and how these axillary buds develop: first at releasing dormancy, and second the development of flower buds. Plant quality of *Phalaenopsis* is mainly determined by the number of flower spikes and number of flowers per flower spike (Dueck et al., 2016). Variation exists in the number of flower spikes that develop per plant. Most research has focussed on flowering in *Phalaenopsis*, and timing of flower induction and flower development can be controlled quite accurately. However, it is still not well understood how the number of flower spikes or the degree of branching can be environmentally regulated. Furthermore, it is unclear how environmental conditions, in particular in the vegetative phase, can contribute to increased plant quality (Runkle, 2019).

2.2.2. Breeding of new varieties in Phalaenopsis

There is large genotypic and phenotypic variation among *Phalaenopsis*. Selection of new varieties is done based on phenotype, by manually choosing cultivars that look nice (e.g. colour, shape and flower number). In breeding, the focus lies on creating either "regular-sized" plants, that have fewer flower spikes with fewer, but larger flowers, or on creating "smaller" plants, that have a higher number of smaller flowers (Figure 1.4). These are sometimes referred to as Grandiflora and Multiflora plant types, respectively (Chen & Lin, 2004). Unfortunately, rates of growth (i.e. biomass accumulation) and development may later appear to be disappointing or deviate otherwise from what was expected. Furthermore, interaction of genotypes with the environment (GxE) occurs. For instance, some genotypes do well under a range of environmental conditions, whereas others do better under a specific environment only.

Breeders aim to match genotype and environment, so they can obtain improved phenotypes (Malosetti et al., 2013). Due to the long growth cycle and GxE interactions of *Phalaenopsis* (e.g. Dueck et al., 2011; Hückstädt & Torre, 2013; Paradiso & De Pascale, 2014), it easily takes several years to select a promising cultivar. Breeding of *Phalaenopsis* is currently lacking knowledge of traits that could function as an (early) indicator for product quality. Breeding could be accelerated by finding selection criteria that allows breeders to discard low quality plants early, or by finding ways to increase rates of growth and development during vegetative growth. Additionally, increased knowledge on GxE interaction in *Phalaenopsis* will allow for better genotype specific climate control and further optimisation of *Phalaenopsis* growth during propagation and cultivation.

2.3. Environmental conditions

2.3.1. Temperature

During commercial cultivation, temperature is the primary determinant for the different *Phalaenopsis* cultivation phases (Blanchard et al., 2007, Anthura, 2017). *Phalaenopsis* plants are grown vegetatively at relatively high temperatures (±28°C) to promote plant growth and leaf initiation (Runkle, 2019). Warm day temperatures in particular prevent premature flowering, regardless of night temperature (Blanchard and Runkle, 2006). *Phalaenopsis* plants can tolerate relatively low night temperatures during vegetative growth (Pollet et al., 2011*a*). Low night temperatures are positively correlated with nocturnal CO_2 uptake in CAM plants (Yamori et al., 2014), although the extent of this effect depends on growth temperature and plant species. In *Phalaenopsis*, lower night temperatures lead to higher malate accumulation, possibly due to a higher contribution of recycled night time respiratory CO_2 (Pollet et al., 2011*b*). During the cooling phase, plants typically are exposed to temperatures between 19-21°C (Paradiso et al., 2012). *Phalaenopsis* has a qualitative vernalization response (Chen et al., 1994). This means that low temperatures (<25°C) are required to release buds from dormancy. Forcing flowering at a lower temperature can result in a higher number of flower spikes and higher number of flowers, but this goes together with delayed plant development and increased time to flowering (Blanchard and Runkle, 2006, Dueck et al., 2016). Exposing vegetative plants to a cooling treatment prematurely can result in delayed beginning of flowering, as well as shorter flower spikes with fewer flowers (Paradiso and De Pascale, 2014). The cooling phase is followed by a

finishing phase at slightly higher temperatures (22°C), which accelerates flower development and outgrowth.

2.3.2. Light

While *Phalaenopsis* is a shade-tolerant plant, increased light intensities during vegetative growth increased leaf area and leaf initiation rate (Konow and Wang, 2001, Dueck et al., 2011, Kromdijk et al., 2012, Hückstädt and Torre, 2013). Lee et al. (2019) showed that plant biomass of vegetative *Phalaenopsis* plants increased when plants were exposed to more light, either via an increased light intensity or via prolonged photoperiod. While CO_2 uptake rates in *Phalaenopsis* saturate at relatively low light intensities, the saturating light intensity increases with increasing temperature (Lootens and Heursel, 1998). Increasing light intensity during the cooling phase can also reduce duration of flower spike development and anthesis of the first flower (Paradiso and De Pascale, 2014). The opposite is also true; when light intensity during the cooling phase is too low, plants will not flower (Wang, 1995). Apical dominance of the vegetative meristem is reduced at low temperatures, but the release of axillary bud dormancy can also be stimulated with light with high a phytochrome photostationary state (PSS) (Dueck et al., 2016). When *Phalaenopsis* plants were placed under treatments differing in spectral composition (% blue light), the effect differed at the two sampling moments and for the two genotypes tested, and no clear patterns were found in e.g. fresh weight, leaf area and pigment composition (Ouzounis et al., 2015). In general, little research has been performed on the effects of spectral composition of the light on *Phalaenopsis* growth and flowering (Runkle, 2019).

2.3.3. CO_2

CO_2 enrichment in the vegetative phase can increase plant growth and leaf initiation (Jin Kim et al., 2017), and can have a delayed effect in increasing flower spike biomass at the end of the flowering phase (Kromwijk et al., 2014). When applied during the entire cultivation period, it can result in an increased number of flower spikes, increased number of flowers, and faster flower development (Endo and Ikushima, 1997, Kromwijk et al., 2014, Trouwborst et al., 2016). However, according to Jin Kim et al. (2017) the long term effect can be adverse, as flower quality was lower due to bud abortion at high levels of CO_2. The effect of CO_2 enrichment is particularly

interesting in the light of CCM in CAM, due the temporal separation of carboxylases. Ambient concentrations of CO_2 are generally believed to be saturating for PEPC in phase I (Winter and Engelbrecht, 1994), but this would not be the case for Rubisco in phases II and IV (Maxwell et al., 1999). CO_2 concentration at the carboxylation site for Rubisco can be as low as 110 ppm during phase IV (Maxwell et al., 1997). CAM species show large variation in their response to CO_2 enriched air, and while CO_2 uptake generally tends to increase, timing of additional CO_2 uptake within the diel cycle differs (Drennan and Nobel, 2000). Nocturnal CO_2 uptake in *Phalaenopsis* can significantly increase when concentration of CO_2 increases (Lootens and Heursel, 1998, Trouwborst et al., 2016), although other studies found contrasting effects (Matschke et al., 1998).

3. Societal implications of studying CAM in *Phalaenopsis*

Water is a renewable but finite source, and reliable water services are no longer available in several parts of the world. Agriculture accounts for 70% of global freshwater withdrawals (Food and Agriculture Organizations, 2012). In a world that is subject to climate change, with rising global temperatures and an increasing frequency of floods and droughts (Naumann et al., 2018), it is important to expand our views and look for solutions that help to make agriculture future-proof. There are several approaches, such as increasing production of current crops by increasing photosynthetic efficiency (e.g. Simkin et al., 2019) or developing crops that have higher water-use efficiency, by bio-engineering the C_4 pathway into rice (International C_4 Rice Consortium, see Von Caemmerer et al., 2012). A third viable option lies in exploring the potential of plants that engage in CAM (Yang et al., 2015). Studying CAM in an environment where factors such as temperature, day length, and light spectrum can be precisely controlled, will help answering outstanding questions on fundamental CAM physiology, e.g. stomatal biology (Males and Griffiths, 2017, Gotoh et al., 2019), mesophyll conductance (Cousins et al., 2020), and ecophysiology (Winter, 2019). Furthermore, experimental data can serve as input for system and/or metabolic network models (Chomthong and Griffiths, 2020, Töpfer et al., 2020), which can be used to increase understanding of CAM even further. Eventually, this knowledge can be used to develop sustainable agricultural systems in which CAM crops can contribute to utilization of currently abandoned lands and help to ensure reliable production

yields. Also in plant breeding, an increased knowledge of physiology can aid in the development of new and improved genotypes with e.g. higher WUE or increased productivity.

4. Aim

In this thesis I aim to increase insight in CAM physiology. For this, I use the economically important *Phalaenopsis* orchid as a case study. By studying a range of *Phalaenopsis* genotypes in a controlled environment, knowledge on the specialized photosynthetic adaption that is CAM can be increased. I will do this by studying the effect of various environmental conditions on different biological and temporal scales, ranging from the diel cycle of CAM phases on leaf level, to growth and development of plants over the entire cultivation period.

5. This thesis

This thesis addresses CAM physiology of *Phalaenopsis* orchids. First, in order to increase insight in CAM physiology and growth, I have developed a conceptual framework (Chapter 2) that formed the foundation for the experimental work. Then, in chapters 3-5, I used *Phalaenopsis* as a case study, and worked on several of the concepts that have been identified in chapter 2 (Figure 1.7).

Chapter 2 describes a conceptual framework that lays the groundwork for mechanistic modelling of CAM crop growth. In this chapter I reviewed current literature on CAM modelling, and discuss the pros and cons of existing models. This knowledge is combined into a conceptual, modular framework. It combines the diel cycle of CAM photosynthesis (such as nocturnal CO_2 uptake and CO_2 refixation during the day) and assimilate allocation among carbon pools (such as storage of starch, sugar, and export for growth), with long-term biomass allocation (towards different plant organs, such as leaves and roots). I discuss the implications of growing CAM plants in a production situation, and give suggestions on how to move forward in order to turn this framework into a quantitative model.

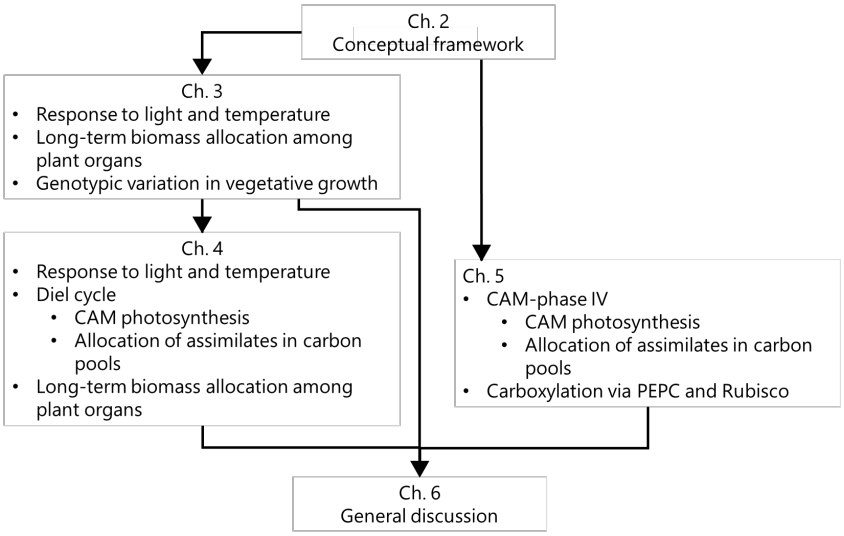

Figure 1.7 Connection of concepts in the conceptual framework that is proposed in chapter 2, to other chapters in this thesis.

Chapter 3 explores genotypic variation in response to temperature and light in *Phalaenopsis* plants. This chapter takes a black-box approach to the conceptual framework and describes the results of two experiments in which I studied the effect of light and temperature in the vegetative phase on vegetative plant growth and development. I also describe the after-effect of applying these treatments in the vegetative phase on generative plant growth and flower quality. Furthermore, I analyse if vegetative traits can be used to predict flowering capacity and quality of the final product. This chapter also illustrates the importance of including a genotypic factor when developing a quantitative model.

In chapter 4, differences that were found in plant growth in response to temperature and light (Chapter 3) were analysed more in-depth. Gas exchange, biochemistry and plant growth were combined to determine the effect of the environment within a diel CAM-cycle. I link data that is collected on different temporal and biological scales; ranging from CO_2 uptake per phase on leaf level, to growth and development of the plant throughout the vegetative phase. Furthermore, I used PCA to understand how two genotypes varied in their response to temperature and light. These results are discussed in context of the conceptual framework of chapter 2. This can help to identify relevant traits for selection in breeding.

Chapter 5 zooms in further on CAM-phase IV. In this chapter, I examine CO_2 uptake via C_3 (Rubisco) and C_4 (PEPC) carboxylation in phase IV in two obligate CAM species; *Phalaenopsis* 'Sacramento' and, the more commonly studied but also horticulturally important, *Kalanchoe blossfeldiana* 'Saja'. It is generally believed that CO_2 uptake in phase IV mainly contributes to growth, because of direct C_3 fixation via Rubisco. That would mean that optimizing and increasing CO_2 uptake in this phase, could make a positive contribution to growth rates. To study the contribution of C_3 and C_4 carboxylation in phase IV, I combined measurements of gas exchange, chlorophyll fluorescence and biochemistry. Additionally, I discuss how having both carboxylases active in phase IV may lead to futile cycling of carbon because of double carboxylation, and hypothesize that this could help to avoid photoinhibition.

The general discussion in chapter 6 synthesizes this thesis. I discuss relevant concepts to be considered when scaling up on biological and temporal scales, from CO_2 uptake and the assimilate pool within a diel cycle on leaf level to growth of the crop over a cultivation cycle. I extend on environmental factors other than temperature and light intensity, as I discuss the effect of red:far-red and CO_2 concentration. Furthermore, I describe how knowledge that is produced in this thesis can be used in Phalaenopsis breeders and growers. Then, I give some considerations for future research on CAM, and end with personal concluding remarks.

Chapter 2

Conceptual framework for plants utilizing crassulacean acid metabolism (CAM): Scaling up from diel photosynthesis cycles at leaf level to crop growth

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Abstract

Crassulacean acid metabolism (CAM) is a specialized photosynthetic pathway. Plants utilizing CAM temporally separate CO_2 uptake during the night from carboxylation via Rubisco during the day. CAM crops can grow on arid and semi-arid lands where production of C_3 and C_4 crops is not or no longer possible. Therefore, CAM crops are important in climate-proof agriculture. There is relatively little knowledge on CAM crops, compared to C_3 and C_4 crops. One way to summarize and improve knowledge on CAM crops is via the use of crop models. Mechanistic models use quantitative descriptions of ecophysiological processes to predict growth and development. Currently, no mechanistic models for CAM exist that combine daily fluctuations in photosynthesis with long term growth and development. Here, we implement current knowledge on CAM physiology into a conceptual framework for crop growth. This approach can combine different biological and temporal scales, and may function as the groundwork for development of a crop model for CAM. Its modular structure combines CAM photosynthesis, assimilate allocation among carbon pools, and biomass allocation towards different plant organs. Existing literature on CAM was reviewed which led to the identification of knowledge gaps, particularly in how captured carbon is processed and allocated, within a leaf and within the plant. We discuss the implications of studying CAM in a production setting, and propose directions for further research.

Keywords: Assimilate allocation, biomass allocation, Crassulacean acid metabolism (CAM), modelling, photosynthesis

1. Introduction

Crassulacean acid metabolism (CAM) is a specialized photosynthetic pathway. CAM plants temporally separate CO_2 uptake during the night from carboxylation via Rubisco during the day. Osmond (1978) was the first to divide the diel cycle of CAM plants in four phases. Phase I takes place during the night, when stomata are open and CO_2 uptake occurs. CO_2 (in the form of HCO_3-) is fixed via PEPC and stored as a C_4 acid in the vacuole; the primary way of CO_2 fixation that is quintessentially CAM. During the day, stomata are closed, malate is decarboxylated and CO_2 becomes available for refixation by Rubisco (phase III). In between, transitional phases II and IV occur, in which the transition from PEPC to Rubisco fixation, and Rubisco to PEPC fixation takes place, respectively. These phases only take place when environmental conditions are favourable. Otherwise, stomata remain closed and the plant may use respiratory CO_2 as carbon source. The regulation of CAM phases is under circadian control, which functions in anticipation of periodic changes in the environment. This can be finetuned by biochemical processes in order to adapt quickly to unexpected fluctuations (Dodd et al., 2002).

CAM land plants have evolved in areas where water is limiting, and can grow in all parts of the world, from hot and dry deserts, to humid tropical forests with intermittent rainfall (Gilman and Edwards, 2020). Because CAM plants keep their stomata closed during (the largest part of) the day, they are very water-conservative and have high water use efficiency (WUE). The annual crop water demand is only 20% of what is needed for C_3 or C_4 crops (Borland et al., 2009). Therefore, CAM crops can potentially grow on arid or semi-arid lands, and in areas that have become unsuitable for C_3 or C_4 crop production (Yang et al., 2015). CAM crops can contribute to utilization of currently abandoned lands, and allow for reliable crop yields under drought conditions.

1.1. Current literature does not focus on agricultural production of CAM plants

It is difficult to capture CAM as a trait. CAM generally exists together with either C_3 or C_4 photosynthesis. Expression of CAM can be induced ontogenetically (CAM will be expressed with aging of photosynthetic tissue), but other species express CAM only when environmental

conditions are stressful (facultative CAM)(For a detailed overview, see e.g. Winter (2019). CAM ornamentals such as *Phalaenopsis* and *Kalanchoe* are at the top of the list of potted plants sold (Flora Holland 2019). CAM crops such as *Agave*, *Aloe*, *Opuntia*, *Ananas comosus* (L.) Merr., and *Vanilla* are grown for food, and as biomass for fuel. While CAM plants represent 6% of all vascular plants, only 0.002% of cropland in the USA is dedicated to CAM crops (Davis et al., 2019). Therefore, research has focussed mainly on C_3 and C_4 plants, even though CAM plants can potentially reach high productivity levels (Nobel, 1991). Studying CAM in an agricultural setting would help in answering questions regarding higher and more efficient production, in the face of both climate change and changes in agricultural demand, without harming the environment (Jones et al., 2017). The number of papers that study the effect of the environment on CAM crop productivity are limited (Owen and Griffiths, 2014, Niechayev et al., 2019*a*). This type of research is very topical and timely in C_3 and C_4 crops, where FACE experiments are used to study the effects of for instance elevated CO_2 and temperature on rice (Cai et al., 2018) and wheat (Eller et al., 2020). Research on how crops respond to a broad range of environmental conditions will increase insight in physiology, while the experimental data can simultaneously function as input for crop models.

1.2. Crop modelling as a tool to increase knowledge on CAM physiology

Crop models are a collection of mathematical algorithms that capture information of agronomy and physiology. They can help to predict responses of crop growth and development to, for instance, climate change and changes in management strategies (Asseng et al., 2014, Peng et al., 2020). Process based crop models are commonly used in modelling agricultural systems of C_3 and C_4 crops. They can include species-specific and/or genotypic characteristics, and parameters that represent crop management practices (Jones et al., 2017)These mechanistic models are designed to calculate the use and allocation of resources in response to climate conditions, water, and nutrient availability. System dynamic models account for individual processes, but also consider coordination and feedback due to interconnection between processes. These models are used to predict yield, and can function as a decision making tool (Peng et al., 2020). Commonly known examples are APSIM (Keating et al., 2003), DSSAT (Jones et al., 2003), GECROS (Yin and van Laar, 2005) and STICS (Brisson et al., 2003). System dynamic

modelling can be captured schematically and can be visualized with state/rate variables (Forrester, 1961). Although models for CAM plants exist, there are none that focus on mechanistically combining different spatial and temporal integration levels. A mechanistic crop model that uses a systems dynamic approach would help to gain insight in what potential CAM crops have, how they would be influenced by environmental conditions, and how plant specific traits play a role in this.

In this chapter, we review existing literature on modelling of CAM, and discussed pros and cons of these models (section 2). We connect this with existing knowledge on CAM physiology into a conceptual, qualitative framework that combines the diel cycle of CAM phases with growth (section 3). The framework aims to combine current knowledge on different spatial and temporal aggregation levels. In order to do so, we took a basic C_3 framework and stepwise expanded it to capture CAM physiology. The conceptual framework provides a base for future development of a quantitative model based on knowledge from plant physiology, rather than equations that only describe plant behaviour. We conclude with describing the implications of studying CAM in a production situation (section 4), followed by suggestions for future research (section 5).

2. Modelling in CAM plants

2.1. EPI models

The first model for CAM plants was developed and introduced by Nobel (1984), and makes use of the empirical-based Environmental Productivity Index (EPI): it combines the input of environmental variables such as light and irrigation to predict productivity of a crop, using a set of descriptive equations. EPI models lack modelling of physiological mechanisms. This type of modelling focusses on the plant as one unit, and does not consider differences throughout the shoot on for instance leaf level. In the last decade, papers have been published that are based on (updated versions of) this model, which seem to give a decent prediction of yield for a range of climate conditions (Garcia-Moya et al., 2011, Owen and Griffiths, 2014, Owen et al., 2016, Niechayev et al., 2019*a*). However, the temporal resolution of an EPI model is low, commonly set to one month. It is therefore limited in its capacity to capture the plasticity of

CAM plants. Because of these constraints and its high integration level, EPI itself is not very useful for understanding growth of CAM plants from a physiological perspective.

2.2. Carbon flux models

Borland (1996), followed by several others (Ceusters et al., 2010, Haider et al., 2012), used gas exchange and metabolite data to calculate the amount and the partitioning of carbohydrates over different competing carbon sinks in CAM. These calculations provide insight in carbohydrate processing and carbon fluxes at different environmental conditions. While referred to as carbon models, we do not consider this modelling in the strict sense, as these calculations cannot be used to make predictions on future crop growth and development. Owen and Griffiths (2013) developed a quantitative system dynamics model for *Kalanchoe daigremontiana*, that accurately captures the plasticity and shifts of different CAM phases. They showed that switching to different phases is largely determined by metabolic regulation. This model simulates carbon assimilation on cell level, which is extrapolated to leaf level. This model focusses on simulation of a diel cycle only. Bartlett et al. (2014) created a model that couples leaf carbon assimilation and CAM carbon fluxes with stomatal conductance (g_s) and a soil-plant-atmosphere-continuum (SPAC) model. The latter allows control of water fluxes in the plant, including plant water capacitance. Their model did not only simulate the four phases of CAM throughout a diel cycle, but was also able to reproduce the effect of soil drying over several days. Combining a leaf carbon model with SPAC is in between EPI and mechanistic modelling. The Bartlett et al. (2014)-model was further developed by Hartzell et al. (2018) who added a component for storage and release of malate, based on an endogenous circadian rhythm. This addition allowed the model to better simulate the effect to a change in the environment, as well as to set limits to the expression of CAM. It can predict g_s, carbon assimilation, and transpiration under well-watered and drought conditions. Very recently, the model was expanded with an EPI section to predict biomass accumulation at a monthly time scale (Hartzell et al., 2021). While this is an important improvement, assumptions for biomass accumulation are simplified and still suffer from the low resolution of EPI. The implications of this will be discussed in section 4.

2.3. Metabolic network models

Cheung et al. (2014) developed a diel flux balance analysis (FBA) model that simulates the flow of metabolites over a day-night cycle, throughout a metabolic network. FBA modelling is done by setting certain constraints and then calculating the minimum or maximum of a function by linear programming. The diel FBA model was initially developed for C_3 leaves, but by adding CAM-specific constraints, such as setting CO_2 uptake rates in the light to zero and having no photorespiration occur during the day, it was able to calculate fluxes in a CAM leaf. Shameer et al. (2018) updated the model with several important features of CAM, In following years, the model was further updated with proton-balancing of the vacuole at night in order to account for the acidification by malic acid storage (Shameer et al., 2018). In a further iteration, it was coupled with gas-exchange data that allows to study the effect of different environmental conditions on leaf-level (Töpfer et al., 2020). These models were used to show that e.g. energetic costs are three-fold higher at night in CAM leaves, compared to C_3 leaves, but that this is counter-balanced by increased efficiency, as CAM plants make use of carbon-concentrating due to malate decarboxylation behind closed stomata during the day. Current FBA models for CAM focus on cell and leaf-level scales, within a diel cycle only. These detailed biochemical models requires further development before they can be used to predict crop growth and yield (Shameer et al., 2018).

2.4. Conclusion

On one hand, there are detailed mechanistic CAM models that look at carbon assimilation within a diel cycle, and how this is affected by a change in environmental conditions. On the other hand, there are EPI models that focus on a higher integration level over a longer time period, but those are based on descriptive calculations only. It is challenging to combine different spatial and temporal levels within one mechanistic model and so far, this has not been done for CAM plants. Creating a crop model for CAM plants requires scaling up from the current physiological models that function on cell and leaf level, to plant and crop level. To mathematically capture quantitative information in a crop model in response to changes in the environment, we first must determine the key processes in the functioning of CAM.

3. Development of a conceptual framework for a mechanistic crop model for CAM crops

Here, we introduce a framework that could function as the groundwork for the development of a mechanistic growth model for CAM crops. As the basis we used a C_3 growth model that is assimilate-driven (Figure 2.1). Step by step, changes relevant for CAM crops will be introduced into this framework, and implications will be discussed. The conceptual framework has a modular approach, and is made up of three modules (Figure 2.2). The first module focuses on CAM photosynthesis and diel changes in physiological and biochemical processes like CO_2 uptake and kinetics of the most important enzymes, such as PEPC (phosphoenolpyruvate carboxylase) and Rubisco (3.1). This module focuses on leaf level physiology within a diel cycle. In the second module of the framework, we describe what is known about assimilates, and how they are apportioned amongst different pools, like those for nocturnal PEP synthesis and growth (3.2). In this module, the focus shifts from leaf to plant level. The third module goes beyond diel patterns and describes biomass partitioning among different plant organs (3.3). This module should be considered long-term, and covers the growth cycle of a crop.

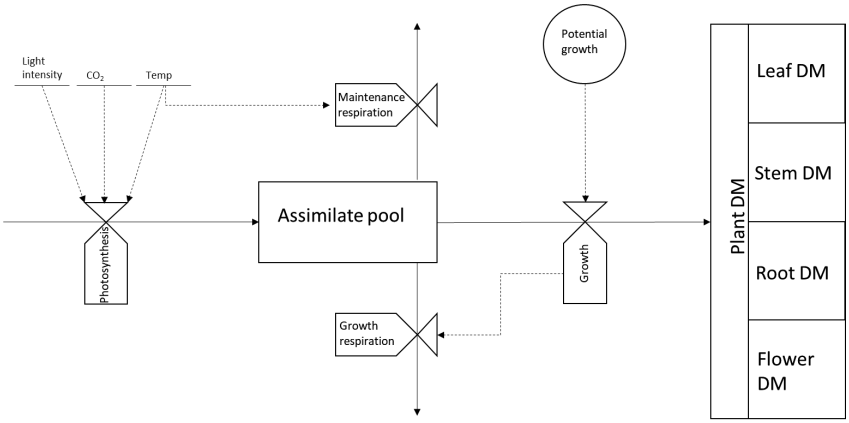

Figure 2.1 Schematic representation of the fundamentals of a C_3 growth model, adapted from van Ittersum et al. (2003). Forrester's (1961) symbols are used: boxes for state variables, valves for rate variables and circles for intermediate variables. Full-line arrows for carbon flows, and dashed-line arrows for information flows. DM: dry matter.

3.1. Module 1: CAM Photosynthesis

3.1.1. Stomatal conductance

Diffusion of CO_2 from the air to the site of carboxylation is subject to several resistances. The boundary layer resistance is assumed to be negligible, in contrast to the high internal resistances within a leaf, that restrict diffusion. Both stomatal conductance (g_s) and mesophyll conductance (g_m) are important limiting factors in CO_2 uptake (Griffiths and Helliker, 2013). While the anatomy of stomata in CAM plants is in principle not different from C_3 plants, g_s in CAM plants is much lower than for C_3 relatives in the same lineage (Males and Griffiths, 2017). Even when stomata are wide open, g_s of CAM plants can be lower than what is found for C_3 plants with closed stomata in the dark (Black and Osmond, 2003). This is due to lower stomatal densities (Males and Griffiths, 2017), even though stomatal pore size is larger (Niechayev et al., 2019*b*). In *Kalanchoe laxiflora* plants restrained from capturing CO_2 at night by silencing the gene that encodes for PEPC, stomatal opening to shifted towards a C_3-like pattern (Boxall et al., 2020). It is believed that C_i is a major driver in stomatal opening, and it is possible to model this response using a bottom-up approach, as Chomthong and Griffiths (2020) suggest. Such an approach does not require detailed mechanistic knowledge to capture stomatal behaviour

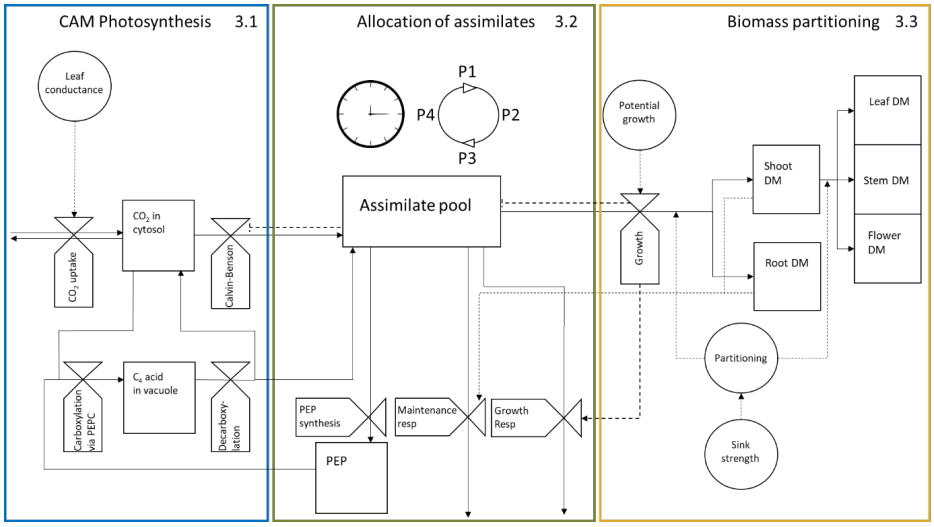

Figure 2.2 Conceptual framework for crassulacean acid metabolism (CAM) crops divided into three modules that can function as the outline for a mechanistic and quantitative model that combines different time scales and integration levels. Forrester's (1961) symbols are used: boxes for state variables, valves for rate variables and circles for intermediate variables. Full-line arrows for carbon flows, and dashed-line arrows for information flows. Abbreviations: PEP, phosphoenolpyruvate; DM, dry matter.

and the pattern of CO_2 uptake in model calculations. However, it does not help in answering outstanding questions on physiology, such as how stomatal aperture is regulated, how their response to environmental stimuli is different from C_3 plants, and how this sensitivity varies among CAM plants (Males and Griffiths, 2017). A better understanding of guard cell metabolism and regulatory processes that drive stomatal opening and closing in CAM plants is still needed.

3.1.2. Mesophyll conductance

Leaf conductance of *Kalanchoe daigremontiana* Hamet et Perr. was amongst the lowest ever recorded (Maxwell et al., 1997). Although g_s in CAM plants is very low, mesophyll factors are considered more important in limiting the rate of nocturnal assimilation (Winter, 1985). A general trait of CAM plants is tissue succulence. This is characterized by thick leaves and large mesophyll cells, which harbour a large vacuole that accommodates nocturnal malate storage (Barrera Zambrano et al., 2014). Combined with tight cell packaging, intracellular air space is reduced, compared to C_3 plants. The amount of intracellular air space in succulent CAM plants can be as low as 5%, which greatly reduces the capacity of internal diffusive CO_2 supply (Nelson and Sage, 2008). Thick leaves, large mesophyll cells and low intercellular air space combined result in low g_m. However, these traits are beneficial for the carbon concentrating mechanism of CAM plants (Nelson and Sage, 2008), and were therefore considered to be correlated. This assumption holds for an individual family such as *Orchidaceae* (Winter and Smith, 1996), but leaf anatomy traits cannot be generalized over all CAM species and lineages (Herrera, 2020). Interestingly, cell size hardly correlates with CAM expression. How mesophyll anatomical traits affect g_m in CAM plants exactly remains unknown. Low g_m in CAM plants reduces the chance of CO_2 leaking during phase II and III, when internal CO_2 is high. During phase I, low g_m could promote capturing of respiratory CO_2 (Borland et al., 2018). g_m changes throughout a diel cycle, and should not be presumed constant. This is due to changes in biochemistry, such as the inhibition of PEPC by malate accumulation (Winter, 1985). Furthermore, the temporal separation of PEPC and Rubisco activity, and their location in either the mesophyll cytosol or chloroplast stroma, respectively, affects the physical component of g_m. Determination of the exact effect requires more measurements under a range of environmental conditions (Tholen et al., 2012). Ideally, a model for CAM should be able to function with different sets of species-

specific parameters, as for instance in the model DSSAT (Jones et al., 2003).

Different approaches are used when modelling leaf conductance, such as using a species-specific maximum of g_s and g_m that scales based on pCO_2 and pH_2O (Owen and Griffiths, 2013), a simplified gas-diffusion model that indirectly accounts for stomatal conductance and can predict water-loss on leaf-level (Töpfer et al., 2020), or a combined value for g_s and g_m based on vapour-pressure deficit (Hartzell et al., 2021). In case of the latter, they used this model to calculate WUE based on a leaf under drought, and extrapolated it to the whole plant. Extrapolation of conductance from leaf to plant should be done with caution, as they are not always correlated, especially under drought stress (Kiwuka, 2020). Both stomatal opening and mesophyll metabolism are under circadian control. This is refined by other endogenous and exogenous stimuli, which are likely to vary throughout a crop. Therefore, modelling leaf conductance would also require temporal parameterization within a crop model. In order to scale up from leaf level to a whole plant, including information on e.g. architectural parameters would be useful. Studying plants under a variety of environmental conditions is needed to quantify the limitations caused by leaf conductance.

3.1.3. Nocturnal carboxylation via PEPC and storage in C_4 acid

During CAM phase I at night, phosphoenolpyruvate carboxylase (PEPC) catalyses atmospheric or respiratory CO_2 (as HCO_3^-) to bind with phosphoenolpyruvate (PEP) into oxaloacetic acid (OAA) which is converted to a C_4 acid, and stored in the vacuole. Storage in the vacuole occurs mainly in the form of malic acid. Besides malate, CO_2 could also be stored in isocitrate or in citrate, which is a tricarboxylic acid (Miszalski et al., 2013). Citrate is part of the tricarboxylic acid cycle (TCA or Krebs cycle) and is synthesized from OAA by the addition of another acetyl group. In total, it contains six carbon compounds compared to four in malate, and could therefore facilitate higher nocturnal accumulation of carbon. Citrate does not contribute to net carbon gain because of its higher energy demand in the light, but can be beneficial, as it contributes to energy dissipation (Lüttge, 2002). Active transport of protons into the vacuole by a H^+-ATPase pump creates an electrochemical gradient, which allows for the influx of the C_4 acid into the vacuole through a malate selective anion channel. C_4 acids accumulate in the vacuole during the uptake of CO_2 at night until the vacuole is filled, which can occupy up to 95% of cell volume.

Nocturnal CO_2 fixation can be limited by PEP substrate availability (Borland and Dodd, 2002) , which is caused by transitory starch or soluble sugar depletion (Dodd et al., 2003). The amount of PEPC does not fluctuate over time, but its activity does, which is controlled via phosphorylation (Nimmo et al., 1984, Chollet et al., 1996). Keeping plants in CO_2 free air for 24 hours strongly reduced CO_2 uptake, although fixation of respiratory CO_2 could still occur. Phosphorylated PEPC is more active and more sensitive to the positive effector Glc6P (Borland and Taybi, 2004, Borland et al., 2016). The inhibitory effect of malate is ten times stronger if PEPC is not phosphorylated (Nimmo, 2000). However, dephosphorylation does not seem to be under circadian control alone, as metabolic regulation can override circadian control (Nimmo, 2000, Dodd et al., 2003). PEPC activity remains upregulated longer in plants that were prevented from accumulating malate (Borland and Taybi, 2004), compared to those with malate accumulation in phase I. Also vacuole size can be a limiting factor in the amount of C_4 acid that can be stored, which can limit the extent of CAM (Winter, 1985, Töpfer et al., 2020). CAM can furthermore be limited by the maximum pH difference across the tonoplast created by proton pumps (Holtum et al., 2005). However, it is more likely that a decrease in malate accumulation rate at the end of the night is caused by other processes. If there is limited energy availability of ATP for proton pumps (Holtum et al., 2005), this might result in accumulation of cytosolic malate, inhibition of PEPC, and thus reduced CO_2 uptake (Borland et al., 1999).

3.1.4. Tonoplast switch from influx to efflux

During phase II, PEPC activity may be prolonged, as dephosphorylation is delayed, while Rubisco activity is being upregulated and light levels are maximal (Maxwell et al., 1997). Having both PEPC and Rubisco active results in the typical 'morning burst' that is seen in CAM CO_2 uptake profiles. Due to the limited activity of Rubisco during phase II, photosynthetic sink strength for electrons is still low, and it takes several hours for electron transport to reach its maximum (Griffiths et al., 2008). Having PEPC still active early during this phase might serve as photoprotection (Roberts et al., 1997). During phase II, the tonoplast switches from influx of malate to efflux. The processes by which this switch occurs are still largely unknown. While vacuolar storage and switch of the tonoplast are essential in the functioning of CAM, even detailed metabolic models assumed fixed values in order to model this properly (Shameer et al., 2018). The uncertainty leads to different approaches to modelling tonoplast fluxes and

carboxylase activation. In most models, the tonoplast switch is set to occur at a fixed time (Owen and Griffiths, 2013). Both Bartlett et al. (2014) and Hartzell et al. (2021) model in- and efflux of the vacuole with a cubic function, based on a circadian order variable that depends on temperature and maximum storage concentration of the vacuole, that in return deactivates proton pumping and allows for malic acid release. This function also takes into account PEPC activity. Modelling in- and efflux of the vacuole using based on physiological control appears to remain difficult, and requires further study.

3.1.5. Decarboxylation of CO_2 and fixation via Calvin-Benson cycle

Stomatal closure marks the start of phase III, in which CO_2 is carboxylated by Rubisco and processed in the CB-cycle. Depending on CAM-subtype, malate in the cytosol is decarboxylated by mitochondrial NAD^+-ME (malic enzyme), cytosolic $NADP^+$-ME, chloroplastic $NADP^+$-ME, cytosolic PEPCK, or a combination of these (Christopher and Holtum, 1996). The remaining 3-C compounds PEP or pyruvate are recovered via gluconeogenesis and stored as either starch or sugar. They can be used as substrate for nocturnal carboxylation in the following night (Christopher and Holtum, 1996). It was long suggested that an increase in C_i at the start of phase III was the signal for stomatal closure. However, plants that were exposed to N_2 air in order to prevent malate accumulation, still closed their stomata in the morning and were unresponsive to reduced external pCO_2 in phase III (Von Caemmerer and Griffiths, 2009). While there is an underlying relation of stomatal aperture with biochemistry, these results suggest a secondary control pathway, such as circadian regulation. Malate decarboxylation rate appears to be driven, either directly or indirectly, by light intensity in CAM-phase III, which results in an earlier start of CAM-phase IV (Barrow and Cockburn, 1982; Hogewoning et al., 2021). Furthermore, temperature and light intensity affect the rate of malate efflux from the vacuole (Grams et al., 1997, Lüttge, 2004). Decarboxylation of stored malate results in high internal CO_2, which was thought to suppress photorespiration. CO_2 concentration during phase III ranges from 800 ppm for *Hoya carnosa,* up to 25.000 ppm for two *Opuntia* species, but the latter seems an exception rather than a rule (Cockburn 1979). These internal concentrations are so high that some CO_2 might even leak out (Friemert et al., 1986). However, the high O_2 concentrations that are also generated during this phase (Niewiadomska and Borland, 2008) suggest photorespiration via Rubisco still might occur. The additional ATP and NADPH that is

needed for the recovery of pyruvate requires extraordinarily high levels of electron transport. In CAM plants, rates of electron transport are well in excess of what is required for CO_2 refixation (Skillman and Winter, 1997), but it is likely that alternative processes such as cyclic electron transport also occur to meet ATP demands (Schöttler et al., 2002). As Rubisco activity increases further during phase III, it reaches a maximum at the beginning of phase IV (Maxwell et al., 1999). When the pool of stored malate is depleted and internal CO_2 concentrations drop, stomata may reopen, indicating the start of phase IV. Phase IV is thought to resemble C_3 photosynthesis the most. This phase allows for additional fixation of CO_2, partially directly into the CB cycle (Dodd et al., 2002). Under natural conditions, Rubisco activity decreases towards the end of the day while PEPC is being upregulated. Having both carboxylases active at the same time might result in high rates of photorespiration, as well as double carboxylation (Maxwell et al., 1999). However, compared to Rubisco, PEPC is not a strong electron sink. Having Rubisco active in phase IV might therefore serve as a photoprotective mechanism, which may outweigh the inefficiency of such futile cycling (van Tongerlo et al., 2020, chapter 5 of this thesis). This concept is not yet accounted for in current models.

CAM has evolved in areas where plants had to adapt to severe drought stress, high light conditions, and/or low atmospheric CO_2 (Edwards, 2019). It is not surprising that most of the research so far has focussed on understanding the mechanisms of inverse stomatal opening, temporal separation of CO_2 fixation by PEPC and Rubisco, and how these processes are regulated. Mechanistic modelling is well underway in leaf level models, and provides a good starting point for a quantitative crop model. However, we strongly advise a holistic approach when systematically studying these detailed processes in CAM plants, in order to accurately capture them in a crop model.

3.2. Module 2: Allocation of assimilates among carbon pools

3.2.1. Sugar and starch in the assimilate pool

Carbohydrate processing and partitioning play a central role in regulating growth and productivity. In CAM plants, the assimilate pool is comprised of carbon that is fixed via CB cycle, and of carbohydrates that are available from the recovery of PEP or pyruvate, which is released from the breakdown of malate. Depending on species, plants store carbohydrates for nocturnal fixation either as sugars in the vacuole, or as starch in the chloroplast (Christopher and Holtum,

1996). It is possible to determine the source of carbon in the assimilate pool, since carbon that is fixed directly via C_3 photosynthesis has lower ^{13}C compared to carbon that was initially fixed by PEPC and stored as malate (Deleens and Garnier-Dardart, 1977). The biggest difference of CAM carbon budgets compared to a C_3 carbon budgets, is that a significant amount of carbon is allocated towards the formation of PEP, the substrate that is needed for nocturnal fixation of CO_2, in addition to conventional allocation of transitory starch to respiration (see e.g. review of Graf and Smith (2011)). CAM plants need to carefully balance carbohydrate availability, which seems to be the limiting factor for nocturnal CO_2 carboxylation in CAM. Sufficient carbohydrates need to be stored in order to have PEP substrate available for nocturnal CO_2 fixation (Borland et al., 2016). The carbohydrate demand for CAM can take up to 20% of a plant's carbon budget (Borland and Dodd, 2002). However, it appeared that the additional cost of CAM does not lead to a significant growth penalty (Shameer et al., 2018). Nobel (1988) already showed that CAM productivity can be as high as what is found for some C_3 crops.

3.2.2. Starch degradation pathways differ between C_3 and CAM plants

Underlying mechanisms to regulate starch turnover are likely similar between plant types, as all enzyme and transport reactions of C_3 plants are also present in CAM plants (Winter et al., 2015). Research on starch turnover pathways has just started in CAM plants. Steps have been made on understanding how growth and starch turnover correlate in C_3 plants, but how this fits in with other metabolic processes and how this is regulated remains to be seen (Stitt and Zeeman, 2012). These correlations have not been studied in CAM plants. Large-scale proteomics analysis of epidermis and mesophyll layers of *Kalanchoe fedstchenkoi* leaves indicated that there is tissue-specific specialization of isozymes in carbohydrate turnover, as well as a diel rescheduling of guard cell starch turnover, when compared to *Arabidopsis* (Abraham et al., 2020). It is unclear how this is controlled in sugar-storing CAM plants. How sugar signalling pathways regulate the import of sucrose during phase III, while restricting export of hexose until the night, requires further study (Antony and Borland, 2009). C_3 plants mainly break down starch via the hydrolytic route, while CAM plants mainly use the phosphorolytic pathway (Borland et al., 2016). The nocturnal rate of starch breakdown is 8.7 times higher in CAM plants compared to C_3. Using the phosphorolytic pathway avoids the need to use ATP-dependent hexokinase to phosphorylate glucose (Shameer et al., 2018). This allows

CAM plants to compensate for the additional costs of malate storage in the vacuole. Using the phosphorolytic pathway allows for a 14-26% energy saving of in using nocturnal ATP in CAM, compared to using the hydrolytic pathway. In C_3 plants there is a slight energy saving of 4-8% when using the hydrolytic pathway, but because the starch turnover rate is much lower, having the most efficient route is a lot less relevant (Shameer et al., 2018). The diversification of the C_3 pathway and of timing of chloroplastic transporters is relatively clear for starch-storing CAM plants, but it is yet unclear if and how vacuolar transporters of sugar-storing CAM plants have diversified (Borland et al., 2016).

3.2.3. Respiratory fluxes in CAM plants

Respiration plays a central role in CAM plants, as the carbon concentrating mechanism to store CO_2 as malate relies on several subprocesses that are part of respiratory metabolism, such as the accumulation and degradation of malate and citrate (Tcherkez, 2017). It is difficult to measure respiration in CAM plants, as CO_2 produced by respiration in CAM plants can be refixed both during the day and the night. The carbon budgeting method of Borland (1996) is very useful in determining the direction and the size of each carbon flux in and out of the assimilate pool. By calculating how much malate accumulated during the night versus the amount of CO_2 that is taken up, Borland (1996) was able to calculate the amount of respiratory CO_2 that was recycled. A later iteration by Ceusters et al. (2008) combined carbon for export out of the leaf and for respiration in one pool, assuming that all carbon that is degraded, but not accounted for by formation of malate, is allocated towards growth and/ or respiration.. Both versions of the model end their calculations at daily export to sinks (Borland, 1996), without considering how exactly this translates into growth. Hartzell et al. (2020) assumed respiration functions in a similar fashion as for C_3/C_4 plants, being a constant fraction of net carbon assimilation, with an additional layer of feedback via temperature or water stress. While CO_2 uptake is so closely linked to respiration in CAM, this approach takes no notice of important processes such as the possibility of recycling respiratory CO_2.

3.2.4. Allocation of assimilates varies with environmental conditions and developmental stage

Reducing light in phase III resulted in an immediate downregulation of malate accumulation in

the following night (Hogewoning et al., 2020). This indicates a direct relation between storage of carbohydrates during the day, and the provision of the substrate PEP during the night. The allocation of assimilates towards respiration, growth, and substrate for nocturnal CO_2 fixation in CAM, can also be linked to the extent to which CAM is expressed. In the C_3/CAM intermediate *Sedum telephium* more starch was stored during the day under drought stress, which allowed the plant to take up more CO_2 during the night (Borland, 1996). The switch to CAM occurred at the cost of export of soluble sugars. In plants grown at low light than at high light, export of assimilates was reduced to almost zero. Developmental stage of organs can also play a role. In obligate CAM plants, CAM photosynthesis always comes to expression regardless of the environment, but photosynthetic tissues often start with C_3 and then mature into CAM photosynthesis over time (Winter, 2019). In developing a quantitative model, it should be taken into account that both environment and developmental stage affect how assimilates are allocated. Variation in allocation of assimilates among carbon pools should be taken account in modelling a single cell or leaf, but when scaling up from leaf to plant or canopy this is even more relevant.

3.2.5. Allocation of assimilates towards competing processes

It is not yet fully understood how the allocation of assimilates between nocturnal storage and export for growth is determined (Borland and Dodd, 2002, Ceusters et al., 2010). Van Tongerlo et al. (2020)(Chapter 5) showed that phase IV uptake might contribute to net assimilation, but not per se in the most energy-efficient way, as futile cycling is likely to occur when both carboxylases are active. Several models that take into account the budgeting of carbon over different carbon pools use fixed parameters that fit the data, rather than a physiological representation. Ceusters et al. (2010) proposed a reservoir model, with pools that fill up with the minimal amount of carbohydrates needed for nocturnal substrate, while only the 'overflow' of this pool is allocated towards direct growth, which then might also feed-forward and stimulate nocturnal carboxylation. This theory goes well with the dogma that phase IV contributes towards growth, but only when substrate pools are filled. Others suggest that assimilates that are directly fixed via Rubisco end up in a different pool than those derived from C_4 carboxylation (Dodd et al., 2002). Winter (2019) suggested that CO_2 taken up and stored in phase IV is used during the following night as substrate for PEP. If CO_2 uptake in phase IV

increases, this would lead to increased nocturnal fixation, which would lead to an increase in growth, and so on. Such mechanisms highlight the importance of a modelling approach that allows for feedback of sub-processes.

In our current framework, besides the nocturnal storage for PEP, respiration is split into two fluxes, being growth and maintenance respiration (Figure 2.2). These should be calculated separately per time step and if possible, per organ. The baseline here should be a mechanistic approach, rather than a descriptive one. To do so, in particular the knowledge gap on respiratory fluxes in CAM requires additional experimental work.

3.3. Module 3: Biomass allocation among organs

3.3.1. Shoot:root ratio

C_3 and C_4 crop models focus on understanding and predicting of crop growth and yield. Crop growth is the result of several sub-processes, like the allocation of assimilates among competing carbon pools, and balancing assimilates among different organs. Until very recently, the focus on growth and productivity was lacking in CAM modelling (Hartzell et al., 2021). The mechanism behind allocation of assimilates towards different plant organs in CAM plants is likely similar to other plant types. In this framework we propose to use most of the principles that are common for C_3/C_4 models. The distribution of assimilates should be based on a functional equilibrium between root and shoot activity. Under steady state conditions and within one developmental phase, there is a balance between water and nutrient absorption, and photosynthesis, but this does not mean that shoot:root partitioning is fixed. Exposing *Mesembryanthemum crystallinum,* a facultative C_3/CAM plant, to salt stress, induces CAM (Haider et al., 2012). They showed that the allocation of assimilates over carbon pools changed, as substrate was needed for nocturnal fixation of CO_2. This reduced export of sugars towards the roots, which affected shoot:root ratio of the plant. Assuming one fixed value for shoot:root ratio as done by Hartzell et al. (2021) might be too simply put, as this balance is subject to change with varying environmental conditions, as well as with the amount of CAM expression (Haider et al., 2012).

3.3.2. Source/sink based allocation

Crop growth models are usually based on source:sink regulated growth, which assumes

allocation of assimilates towards different organs based on their sink strength (Yin and van Laar, 2005). Source strength is the rate of production of substrates for growth, for instance, coming from photosynthesis, or from reserves of carbohydrates in storage organs. Sink strength of an organ indicates the competitive ability of that organ to attract assimilates. First, the maximum potential growth of growing organs under conditions of non-limiting assimilate supply has to be defined, which determines the potential demand or sink strength of tissues to accumulate assimilates (Marcelis, 1996). Source/sink strength is in its most simple form scaled to location of the organ on the plant. For example, a leaf that is positioned lower in the crop is older, and will likely function as a net source, after assimilates needed for maintenance respiration are withdrawn. Younger leaves function mainly as net sinks, and assimilates will be transported to those organs, instead of away. Existing crop models mainly operate based on the classic assumption of source-limited growth (Körner, 2015). This assumption means that the requirement of the plant for assimilates is higher than what is obtained, therefore, all assimilates can be used. Partitioning of assimilates is primarily based on sink strength of different organs. So within the shoot, assimilates are allocated towards different plant organs, like leaves, stems, and in the generative phase, flowers and fruits (Figure 2.2). Other processes, such as the feedback on growth respiration, nutrient balance and allocation of N within the plant should be accounted for. The role of these processes in CAM plants, how they affect the expression of CAM, and how these processes in return are affected by different environmental conditions, should be studied in more depth before they can be quantified within a model.

3.3.3. Mechanistic crop modelling of CAM

EPI models can be used to predict whole plant biomass or yield under different environmental conditions (Nobel, 1984 and others), but this type of modelling is not mechanistic. Two important aspects should be considered when scaling up mechanistic modelling from leaf to plant. First and foremost, the whole plant must be considered. That means: consideration of allocation of assimilates over different plant organs, which have different sink strengths and different requirements. Taking the whole plant into account is particularly important in CAM plants, where the expression of CAM can greatly vary throughout the plant, with age, and with environmental conditions (Winter, 2019). Hartzell et al. (2021) were the first to combine short and long-term growth within one model. This approach seems to function in areas that are

well-watered and where light is not limiting. However, the assumptions they make to predict long-term growth, such as a fixed shoot:root ratio and fixed rate of respiration, may have contributed to the overestimations of productivity up to 40%, in areas where climate conditions are different. In developing a quantitative model for CAM plants, the plants' architecture should also be considered. Light interception varies greatly within one plant (Zotz et al., 2002, Lin and Hsu, 2004), creating a spectrum of microclimates. This affects allocation of assimilates among carbon pools, and allocation of biomass among plant organs.

The framework that is proposed here consists of three modules, that describe key processes in CAM growth. It can function as the basis for a quantitative model that combines knowledge on detailed short-term mechanistic models with long-term growth modelling, while combining different temporal and spatial integration levels (Figure 2.2).

4. CAM in a production situation

The model of Hartzell et al. (2021) greatly overestimated biomass accumulation of *Opuntia* sp. in regions where light conditions were lower than at the validation site. Predictions of biomass accumulation under rainfed conditions (areas with intermittent rain fall) were also much higher than what was observed for these regions. They state that, because their predicted values are much higher than reported, there is ample room for improvement, if planting density and fertilization would be optimized. This means that the predictions are closer to potential yield, than actual yield (van Ittersum et al., 2003). However, it could be that biomass was overrated because they only looked at mature plants, or because they used a fixed, average value for partitioning of biomass among organs, which may be an oversimplification. CAM has evolved several times in areas where plants had to adapt to environmental conditions that would be debilitating otherwise (Edwards, 2019). Developing a model that can accurately predict CAM plant growth at non-limiting conditions may not be so useful for crops where optimizing the production situation is hampered by one or several limiting and reducing factors (van Ittersum et al., 2003). On the other hand, when growing CAM plants in a high-tech agricultural setting like a greenhouse, environmental conditions can be accurately controlled and optimized. It is not clear how this affects the functioning of CAM, as can be seen from the number of studies conducted on CAM species grown in greenhouses such as *Phalaenopsis* (e.g. Blanchard and

Runkle, 2006, Trouwborst et al., 2018, Ceusters et al., 2019). A plethora of questions remain unanswered and there is a lot to learn in CAM-research, like; what exactly determines maximum daily CO_2 uptake? How fast can crops adapt to changes in the environment, and how are these changes regulated? It is worth investigating whether and how some of the common CAM-dogmas hold up. It is often said that, 'when environmental conditions are favourable, transitional phases II and IV can contribute significantly to plant growth', because direct C_3 fixation can occur. In an environment that can be completely optimized and where factors such as day length (Lee et al., 2019) and light quality can be controlled (Gotoh et al., 2019), it remains to be seen whether statements like these still hold. Several studies already suggested otherwise (Ceusters et al., 2010; van Tongerlo, 2020; Hogewoning et al., 2020). Töpfer et al., (2020) also showed that, simulated on leaf level, CAM can be beneficial in temperate climates with lower light conditions. While the water saving potential is lower than in hot and dry environments with ample light, it can still be significant. The research on CAM in temperate climates opens up a whole new area for CAM research.

5. Future research

The framework proposed (Figure 2.2) contains all building blocks that are needed in order to advance mechanistic modelling of CAM within a leaf, to mechanistic modelling of CAM crops. Focussing on the strength of CAM crops rather than their limitations should be one of the pillars of future-proof and climate-robust agriculture. To advance the framework that is proposed here, the different modules require parameterization, and in general more research is needed that studies CAM plants as a whole. CAM photosynthesis (carbon gain) within the leaf is relatively well-studied, but there are still knowledge gaps when it comes to how this carbon is processed and allocated among different carbon pools, within a leaf and within the plant. An example of such a knowledge gap is the functioning of respiration in CAM plants (Tcherkez, 2017).

Scaling up to plant growth over a longer period is now mainly based on what is known from other crop models, but this functioning should be validated in trials with CAM plants. Next steps would be to adapt the model in such a way that it can quantitatively predict crop growth in response different environmental conditions, both on a short- and long-term scale. This can be done partially by retrieving data from literature, but it would also require more

controlled experimental work and data collection. Existing models can be implemented in this framework and function as a basis, but need to be developed further in order to scale up to plant level. In order to make such a model broadly applicable, the setup should be easily adaptable for various species. The goal of this is twofold: it can help to increase yield if plants are grown under optimal environmental conditions, but when able to predict the effects of climate change it can help to mitigate and adapt crop production systems with being more resilient in a hotter and drier world, without harming the environment (Yang et al., 2015, Owen et al., 2016, Jones et al., 2017, Davis et al., 2019).

Chapter 3

Vegetative traits can predict flowering quality in Phalaenopsis orchids despite large genotypic variation in response to light and temperature

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Abstract

Phalaenopsis is an economically important horticultural ornamental, but its growth is slow and costly. The vegetative cultivation phase is long and required to ensure sufficient plant size. This is needed to develop high quality flowering plants. We studied the effects of temperature (27 or 31 °C) and light intensity (60 or 140 µmol m^{-2} s^{-1}) on plant growth and development during the vegetative cultivation phase in two experiments, with respectively 19 and 14 genotypes. Furthermore, the after-effects of treatments applied during vegetative growth on flowering traits were determined. Increasing light intensity in the vegetative phase accelerated both vegetative plant growth and development. Increasing temperature accelerated vegetative leaf appearance rate, but strongly reduced plant and root biomass accumulation when temperatures were too high. Flowering was greatly affected by treatments applied during vegetative growth, and increased light and temperature increased number of flower spikes, and number of flowers and buds. Genotypic variation was large in *Phalaenopsis*, especially in traits related to flowering, thus care is needed when generalising results based on a limited number of cultivars. Plant biomass and number of leaves during vegetative growth were positively correlated with flowering quality. These traits can be used as an early predictor for flowering capacity and quality of the final product. Additionally, this knowledge can be used to improve selection of new cultivars.

Keywords: Genotype x environment (GxE), genotypic variation, growth analysis, flowering quality, orchid, *Phalaenopsis*

1. Introduction

Phalaenopsis is an economically important horticultural crop, which is cultivated either as potted ornamental or as cut flower. The potted *Phalaenopsis* accounts for 19 and 32% of the potted plant sales in the USA and Europe, respectively (Davis et al., 2019). Growth of *Phalaenopsis* is slow and therefore costly. The natural habitat of *Phalaenopsis* is evergreen forests in tropical and subtropical Asia, characterized by relatively constant humid, warm and relatively shaded conditions. While seasonality hardly occurs in these forests, variations in environmental conditions are sufficient to induce flowering (Christenson, 2001). In commercial cultivation, temperature is the main determinant for different *Phalaenopsis* cultivation phases (Blanchard et al., 2007, Anthura, 2017). Plant development is defined by the rate at which organs (e.g. leaves and flowers) are initiated and appear (Atkinson and Porter, 1996). An increase in temperature, up to a certain optimum, increases plant development rates and thus affects duration of each cultivation phase.

Phalaenopsis cultivation can be divided in three separate phases. The vegetative phase of *Phalaenopsis* is the longest phase, which takes 50-70 weeks on average, measured from the moment that plants move to the greenhouse after propagation in the lab (Paradiso and De Pascale, 2014, Anthura, 2017). During this phase, *Phalaenopsis* is grown at high temperatures (≥28°C) which promotes leaf initiation and outgrowth, and inhibit flowering (Runkle, 2019). Flowering in *Phalaenopsis* is mainly temperature-controlled, and temperatures below 25°C induce flowering (Dueck et al., 2016). In practice, plants in the flower induction phase are exposed to temperatures between 19-21°C for 6-9 weeks (Paradiso et al., 2012, Runkle, 2019). This phase is followed by the flowering phase which lasts approximately 8-10 weeks, in which plants are exposed to higher temperatures (approximately 22°C) to accelerate flower development (Paradiso et al., 2012, Dueck et al., 2016). Flower induction and flower outgrowth can be relatively well controlled, and most of the research so far has focussed on induction and the process of flowering itself, ranging from environmental factors (Sakanishi et al., 1980, Wang, 1995, Konow and Wang, 2001, Lee et al., 2019) and hormonal control (Chen et al., 1994, Blanchard and Runkle, 2008) to understanding of the genetic pathways involved in flower development (Wang et al., 2017, 2019). Although *Phalaenopsis* plants are grown for their flowers, the vegetative cultivation phase is important to ensure sufficient plant size. It is

commonly assumed that this is necessary to develop multiple flower spikes and high quality flowers, which increase the plant's economic value (Dueck et al., 2016). Per *Phalaenopsis* leaf, two undifferentiated, dormant axillary buds are present. Under favourable environmental conditions, the upper bud can develop into a flower spike once the plant has matured (Hsiao et al., 2011). Therefore, number of leaves is considered an important indicator during vegetative cultivation (Sakanishi et al., 1980, Paradiso and De Pascale, 2014), as flowering potential is thought to increase with number of leaves. Previous studies on *Phalaenopsis* showed that increasing temperature from 28°C to 31°C in the vegetative phase the number of leaves increased, although this did not always result in increased leaf area (Dueck et al., 2011). Reducing nocturnal temperature resulted in less leaves, lower leaf area and reduced biomass accumulation (Pollet et al., 2011*a*).

Phalaenopsis plants generally require low to medium light levels. Saturating light levels are in the range of 130-200 µmol m^{-2} s^{-1}, depending on plant stage, temperature and cultivar (Lootens and Heursel, 1998, Lee et al., 2019). Because of these low intensities, light is considered of secondary importance by some, when compared to temperature (Lee et al., 2019). However, several studies on *Phalaenopsis* showed that increased irradiance during the vegetative phase resulted in increased leaf initiation rate and leaf area (Konow and Wang, 2001, Dueck et al., 2011, Lee et al., 2019). These traits are also affected by photoperiod and daily light integral, which promoted leaf growth, leaf initiation rate and biomass accumulation (Lee et al., 2019, Runkle, 2019). While a relatively low temperature is considered the main factor for flower induction, a sufficient level of irradiance is also needed for flowering. When light intensities are too low, time to flowering is delayed (Kataoka et al., 2004) or even completely absent (Wang, 1995). Vice versa, higher light intensities in the flowering phase resulted in a reduced time to visible flower spike (flower induction), a higher number of flower spikes, and a higher number of flowers (Konow and Wang, 2001, Dueck et al., 2011, Lee et al., 2019). Time to visible flower spike was positively correlated with higher levels of soluble sugars (Kataoka et al., 2004, Lee et al., 2020), which suggests that there might be a role for carbohydrates in number of days to spiking.

Thus, both light and temperature are important for various key processes in *Phalaenopsis*, and the two factors interact in ways that are yet poorly understood. To provide insight in how underlying traits contribute to growth and development in *Phalaenopsis*, and

how they correlate, a hierarchical component analysis can be used (Higashide and Heuvelink, 2009). It helps to systematically study how these components are affected by changes in environmental factors and their interaction. This method has been applied on several other crop species, such as tomato (Higashide and Heuvelink, 2009), *Anthurium* (Li et al., 2014), and wheat and rice (Cai et al., 2016), and can be used to find desirable characteristics that contribute to either a reduction of vegetative growth time and/or increased plant quality.

The number of studies on young *Phalaenopsis* plants is limited, but considering the lengthiness of the vegetative phase, an improvement might rapidly increase the economy of the cultivation cycle. The need to expand knowledge on young plants is also recognized by e.g. Runkle (Runkle, 2019), who calls for more well-described studies with detailed information on temperature, light intensity and spectrum. Optimizing climate conditions in the vegetative phase might result in a reduction of cultivation time and/or in higher quality plants. In addition, there are indications that genotypic variability is significant in these responses. For instance, Dueck et al. (2011) and Hückstädt and Torre (2013) observed genotypic variation in the response of leaf initiation rates, leaf area increase and dry matter accumulation to light intensity. Genotypic variability is also found for the after-effects of treatments in the vegetative phase on flowering (Hückstädt and Torre, 2013), and while recognized by others (Paradiso and De Pascale, 2014, Runkle, 2019) this variation to date remains largely unexplored. Genotypic variability and specific needs are also ignored in practice, where different genotypes are grown in one greenhouse under identical climate conditions.

This study aims to determine the effects of light and temperature on growth and development of *Phalaenopsis* in the vegetative phase, and how treatments in the vegetative phase affect flower induction and flower outgrowth, on a large set of genotypes. We hypothesize that by finding the right combination of temperature and light for *Phalaenopsis* plant growth in the vegetative and flowering phase, we can positively affect vegetative traits such as leaf initiation and dry matter production, which will increase yield and plant quality. We quantified the contribution of underlying components of plant growth, to determine how these traits correlate with each other. To determine genotypic variation in the vegetative growth response to light, temperature and their interaction, we conducted two experiments with a broad range of genotypes (19 and 14). The latter experiment (with 14 genotypes) was combined with a follow-up experiment to study the after-effects of light and temperature in

the vegetative phase on flower spike growth and quality. Gaining insight in genotypic variability and underlying traits can help to optimize vegetative growth and possible shorten the vegetative cultivation phase. Additionally, this knowledge can be used to improve selection of new cultivars.

2. Material and Methods

2.1. Plant material

Vegetative *Phalaenopsis* plants were grown in a Venlo type greenhouse (Bleiswijk, The Netherlands) for 20 weeks after propagation in the lab, before they were transferred to 12 cm transparent pots filled with coconut bark, before they were used in two separate experiments. Genotypes used in this study were provided by breeding company Anthura (Bleiswijk, The Netherlands). Breeding in *Phalaenopsis* focusses on creating either regular sized plants with fewer, but larger flowers and buds (Figure 3.1A), or smaller plants with a high number of small flowers (Figure 3.1B)(sometimes referred to as Grandiflora and Multiflora plant types, respectively (Chen and Lin, 2004). In experiment I, 13 Grandiflora and 6 Multiflora genotypes were used, in experiment II, 11 Grandiflora and 3 Multiflora genotypes were used, and the selected genotypes consisted of plants with a variety of flower colours, growth rates, and tendencies to carry one or multiple flower spikes. For detailed information on genotypic

Figure 3.1. Representative phenotype of *Phalaenopsis* plant types Grandiflora (A) and Multiflora (B).

similarity and description of phenotypes, see supplemental information S3.3 and S3.4. Genotypes used in experiment II are a subset of experiment I, plus one additional genotype.

2.2. Experimental setup

2.2.1. Vegetative phase (Experiment I and II)

Two separate experiments were conducted, but their experimental setup was largely similar (Table 3.1). Experiment I was conducted at Wageningen University & Research in Wageningen, The Netherlands with vegetative plants that were 9 weeks old after transfer to 12 cm pots. Experiment II was conducted at the *Phalaenopsis* breeding company Anthura in Bleiswijk, The Netherlands with vegetative plants that were grown for 5 weeks after transfer to 12 cm pots.

Table 3.1. Overview of experimental set-up and growth conditions, highlighting differences between setup of vegetative experiment I and II

	Experiment I	Experiment II
Experimental setup		
Number of genotypes	19	14
Transfer to climate chamber	May	February
Duration experiment (weeks)	19	15
Settings in climate chamber		
Temperature (day and night) (°C)	27	26
	31	30
Plant density (plants m^{-2})	80 (9 weeks)	80
	55 (10 weeks)	
CO_2 concentration (ppm)	500	800
Watering interval (days)	5	7

Plants were illuminated for 14 hours per day by red/white, and far-red LED modules (Philips LED production module deep red/white and GreenPower LED research module Far Red; Signify, Eindhoven, The Netherlands) at a PPFD of either 60 or 140 µmol m^{-2} s^{-1} and additional far-red of 10 or 23 µmol m^{-2} s^{-1}, respectively. For a representative spectrum, see supplemental information S3.1. Here, red light is defined as light between 600 and 700 nm, and far-red as light between 700-800 nm, which resulted in a R:FR ratio of approximately 1.2, or a phytochrome photostationary state of 0.83 (Sager et al., 1988). Vapour-pressure deficit (VPD) of the air was set at 1 kPa for all treatments. Plants were watered with nutrient solution with an EC 1.2 mS cm^{-1} and pH of 5.7 (Supplemental information S3.2).

Per experiment, two climate chambers were used, corresponding with temperature treatments. In experiment I, light treatments were replicated three times, but in experiment II,

light treatments were replicated twice. For destructive harvest, 5-7 plants were used per compartment, per genotype. In experiment II, replications in one compartment were treated as being independent. Vegetative plants were destructively harvested after 19 weeks (experiment I) or 15 weeks (experiment II).

In both experiments an initial destructive harvest was conducted (n=15). Number of leaves and roots, leaf area (LI-3100, LiCor, Lincoln, USA), and dry weight of shoot and root were determined. Roots were cut off as close to the stem as possible, and any substrate material that was still attached to the roots was removed. The leaves were then carefully peeled off so the stem remained intact. In the initial harvest, the stem was combined with leaves for dry weight measurements. For all dry weight measurements, plant material was dried for at least 48 hours at 80°C. When plants were transferred to climate chambers, the youngest fully grown leaf of each plant was marked with a clothespin. All leaves that appeared after the leaf with the clothespin were considered new leaves and counted as such. In the final destructive harvest, dry weights of leaves, stem and roots as well as leaf area and number of leaves were determined. Relative growth rate (RGR, g d^{-1}) was calculated according to Eq 3.1, where W represents weight of the plant and t time of harvest. T1 is the initial harvest before start of the experiment. For vegetative plants, this was based on total plant dry weight, but for flowering plants RGR was based on shoot dry weight only. Leaf mass area (LMA, kg m^{-2}) is the ratio of leaf mass to leaf area, and was calculated using all leaves per plant.

$$RGR = (ln(W2) - ln(W1))/(t2 - t1) \qquad \text{Eq 3.1.}$$

2.2.2. Generative phase (continuation of experiment II)

Remaining plants from experiment II continued to grow vegetatively at a lower plant density (60 plants m^{-2}) for another eight weeks, before they were moved to the greenhouse (august). Plants were placed at a continuous 19°C (day and night), at a set CO_2 concentration of 500 ppm, VPD of 0.81 kPa at an average daily light integral (DLI) of 7.5 mol m^{-2} day^{-1}. To achieve a sufficient daily light integral within a 15 hour day, high pressure sodium (HPS) lighting was switched on towards the end of the day. Plant density was 50 plants per m^{-2}, and watering was done every 5-6 days. After eight weeks of flower induction, temperature was set to continuous 20°C, CO_2 concentration to 650 ppm, while DLI, VPD and watering schedule remained similar.

Supplemental lighting was applied both at the beginning and the end of the day. Plants of a genotype were harvested when approximately 2/3 of the plants of that genotype had two open flowers per plant (n=10). This is called the consumer-ready stage. At this developmental stage plants would normally be sold, and is therefore a good moment to determine plant quality. This means plants were harvested in a physiologically similar developmental stage, rather than an identical point in time. The first genotype was harvested 15 weeks after transfer to the greenhouse, while the last genotype was harvested after 21 weeks. The number of flower spikes, open flowers, buds, total number of leaves, as well as number of new leaves since the start of the experiment, were counted. Leaf area was determined, as well as dry weight of leaves, using the same method as previously described. To calculate RGR of flowering plants, the number of days until harvest per genotype was used.

2.3. Statistical analysis

Data were analysed using linear mixed-effect models using R version 3.6.1 (R Core Team, 2019) with package lme4 (Bates et al., 2015). In the analysis of experiment I we assumed temperature, alongside light intensity, was replicated independently . In experiment II, both temperature and light were assumed to be replicated independently, and individual plants were treated as independent replicates. The assumption of independent replication of temperature may have underestimated random variance. Residual plot and qqplot were used to determine if assumptions for normality and homogeneity were met. Correlations were tested using Pearson's correlation coefficient, and visualisation of matrices was done using R-package corrplot v0.85 (Wei and Simko, 2018).

3. Results

3.1. Vegetative phase (experiment I and II)

The response to light was very similar in both experiments. Total plant biomass increased with an increase in light intensity (Figure 3.2A,B). While both shoot and root dry weight were higher at a higher light intensity, the effect on root dry weight was larger, resulting in a lower shoot:root ratio at increased light. In contrast, a higher temperature resulted in higher shoot:root ratio. Interestingly, in experiment I, root dry weight was the only trait for which the

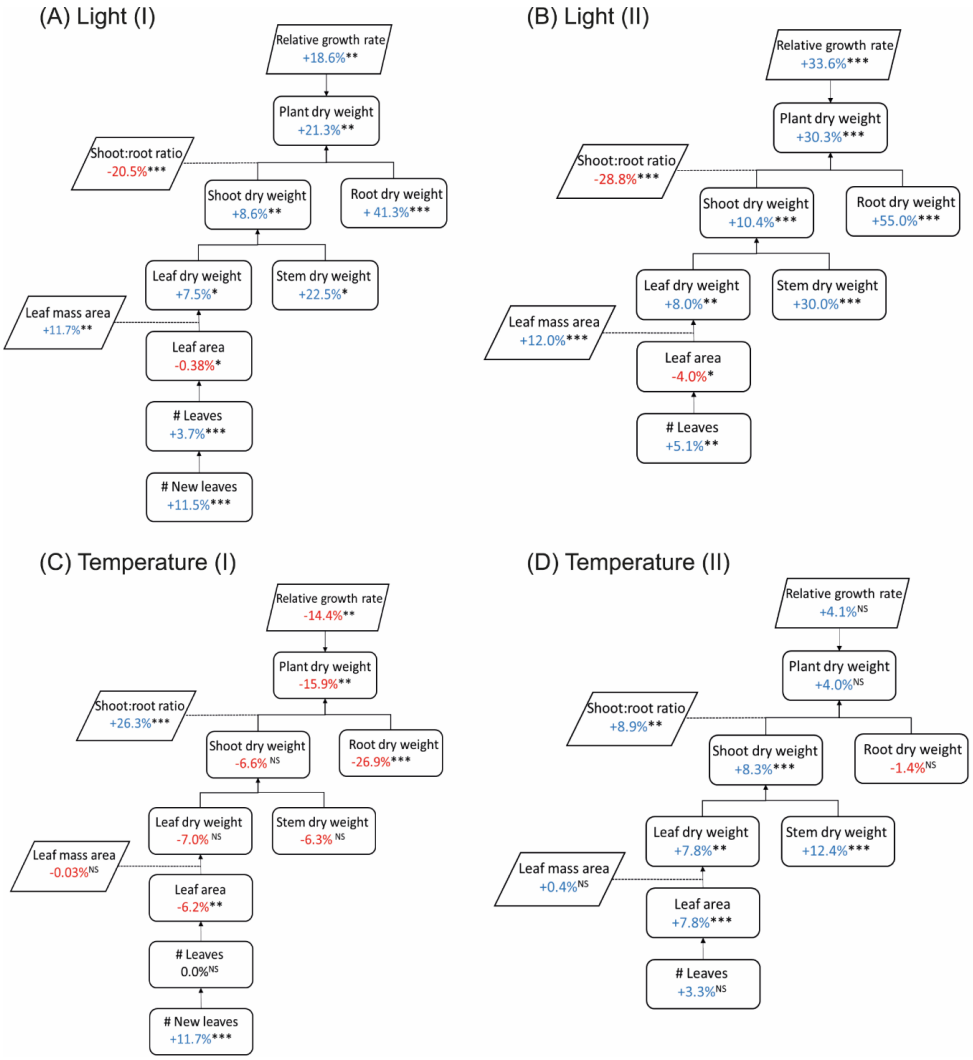


Figure 3.2 Trait component analysis of vegetative Phalaenopsis plants. Main effects of light (A, B) and temperature (C, D). Plants were grown in climate chambers under LED lighting for 14 hours per day at a PPFD of 60 or 140 µmol m^{-2} s^{-1}. In experiment I (A,C; n=3; 5-7 plants per statistical replicate per genotype), plants of 19 genotypes were grown for 19 weeks at either 27°C or 31°C. In experiment II (B,D; n=5; per genotype) 14 genotypes were grown for 15 weeks at either 26°C or 30°C. Data is averaged over genotypes, percentages represent average change per trait to reference light intensity (60 µmol m^{-2} s^{-1}; A, B) or temperature (27°C, C; 26°C, D). NS, *,**,*** are not significant or significant at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

magnitude of the effect of light was temperature-dependent (2-way interaction), being larger at 27°C than at 31°C. In experiment II, this was only the case for stem dry weight. High light intensity resulted in lower leaf area. Number of newly formed leaves, which is considered the most important characteristic needed to shorten growth time, was higher at high light, but was

not affected by temperature. It appeared that leaves of plants grown at a combination of high light and high temperature were smaller, but plants had more leaves. The opposite is true for plants grown under the combination of low light and a lower temperature, which had fewer, but larger leaves.

Averaged over all genotypes together, total plant dry weight and RGR in experiment I were lower at 31°C than at 27°C (Figure 3.2C). The response in plant biomass was mainly due to the decrease in root dry weight at higher temperature. Shoot dry weight, composed of leaf and stem dry weight, was not significantly affected by temperature. There was no effect of temperature on plant dry weight or RGR during the vegetative phase in experiment II (Figure 3.2D). However, shoot dry weight (due to both leaf and stem dry weight) increased with temperature, while root dry weight was not affected. This resulted in an increased shoot:root ratio in both experiments. In experiment I, the leaf area of plants decreased with an increase in temperature, while number of new leaves increased, although the total number of leaves did not. In experiment II, the number of leaves did not change with temperature either. However, in contrast to experiment I, an increase in temperature resulted in a higher leaf area.

A separate analysis of the data that included only those genotypes that were present in both experiments was conducted, to make sure that the differences found between the experiments were not caused by the difference in genotypes that were used. Light treatments in both experiments were identical, and the analysis on the main effect of light indeed led to the same conclusion as previously described. Temperature treatments differed 1°C between experiment I and II. Using the same set of genotypes, there was a significant effect of temperature on shoot dry weight in experiment I, and on number of leaves in experiment II, which was different compared to the analysis of the complete genotype sets. Other traits were not affected compared to the previous analysis.

3.2. Generative phase (Experiment II)

Increasing light intensity from 60 to 140 µmol m^{-2} s^{-1} in the vegetative phase resulted in an increased RGR, which was reflected in both an increased leaf dry weight as well as increased flower spike dry weight (Figure 3.3A) at the end of the flowering phase. An interaction with temperature occurred for flower spike dry weight (2-way interaction), as increasing temperature increased flower spike dry weight at low light, but not at high light. Additionally,

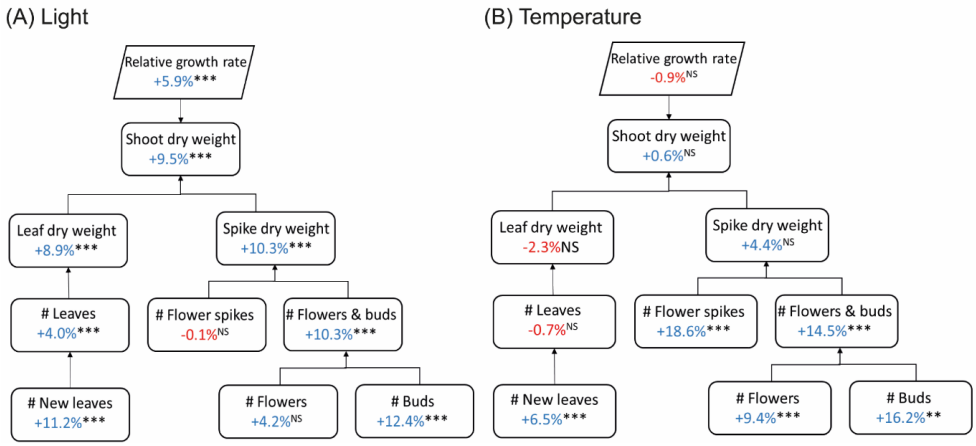


Figure 3.3 Trait component analysis of flowering *Phalaenopsis* plants. Effects of light (A) and temperature (B) treatments applied during the vegetative phase. During the vegetative phase, plants were grown in climate chambers for 23 weeks at either 26°C or 30°C, and a PPFD of 60 or 140 µmol m^{-2} s^{-1} for 14 hours per day. Plants from all treatments were then moved to the greenhouse for flower induction (8 weeks) and subsequent flowering phase. Plants of a genotype were harvested, when 2/3 reached the consumer-ready stage, defined as plants having two open flowers per plant (15-21 weeks, depending on genotype). Percentages represent average change per trait to either vegetative reference temperature (26°C) or light intensity (60 µmol m^{-2} s^{-1}). NS, *,**,*** are not significant or significant at $p<0.05$, $p<0.01$ and $p<0.001$, respectively (n=10 per genotype).

increased light resulted in an increased flower spike quality, defined by the total number of flowers and buds per spike. The increase in number of flowers and buds was due to an increased number of buds. Furthermore, an increased light intensity resulted in a higher leaf number. Similar to spike dry weight, the effect light intensity on number of flower spikes interacted with temperature. An increase in temperature always resulted in a higher number of flower spikes, but this effect was larger for plants grown at low light compared to plants grown at high light during the vegetative phase. There was no main effect of temperature treatments applied during the vegetative stage on biomass-related traits. RGR, shoot and leaf dry weight of the flowering plants were not significantly different (Figure 3.3B). Flower spike dry weight per plant was slightly higher with increased temperature at the end of the flowering phase, although not statistically different. The number of flower spikes did increase. Increasing temperature during the vegetative stage led to a higher number of new leaves for all genotypes in the flowering stage , as well as to a higher number of flowers and buds per plant.

3.3. Genotypic variability

The above results are averaged over all genotypes. However, genotypes varied in growth and development, and their individual response to either light or temperature differed, as interaction between genotypes and environmental conditions occurred (Table 3.2). Significant genotype by environment interactions reflected genotypic variation in the magnitude of responses, and in directionality (Figure 3.4 and Figure 3.5). As described previously, the directionality of the effect of light in both vegetative experiments was comparable, and this was also the case for the range of genotypic variation (Figure 3.4). While stem dry weight stands out as a trait with large genotypic variation, this is probably due to measurement errors, not to a much higher level of variability and will not be considered in further analyses. In both experiments, least genotypic variation in response to light was found for leaf mass area. Largest variation was found for RGR, which varied over 50% between genotypes.

Interestingly, while directionality in response to temperature varies between experiment I and II, the range of genotypic variation was similar (Figure 3.5), but in experiment II this variation was often not significantly different between genotypes, while this was the case in experiment I (Table 3.2). In both experiments, least genotypic variation was found for leaf mass area, making it the most stable trait in *Phalaenopsis*. In response to temperature, number of new leaves formed showed largest genotypic variation in experiment I, which varied almost 50% between genotypes. This trait was not measured in experiment II, where largest variation was found in root dry weight, which varied over 40% between genotypes. Overall, genotypic variation was larger in response to light than to temperature. During the flowering phase genotypic variation also occurred, as well as interaction with the environment of the vegetative stage (Table 3.3). More light led to more genotypic variation in the vegetative phase, which can in part be explained by differences between Grandiflora and Multiflora plant types. Genotypic variation for all traits except number of new leaves could be explained by variation between these two plant types. Number of flowers and buds were much more affected by light in Multiflora plants, than was the case for Grandiflora plants (Supplemental information S3.5). The same was true for a change in temperature when the two plant types were compared (Table 3.2). This was mainly due to a change in number of buds, and not to number of flowers. For the other traits there was no difference between plant types in interaction with the environment. Interaction with the environment mostly came down to variation in the response

of individual genotypes, and not to variation between plant types of Grandiflora and Multiflora. Genotypic variation was also apparent in non-flowering traits at the end of the flowering phase, such as leaf dry weight. However, temperature had a larger effect on flower spike dry weight, and even more so on number of flower spikes (Figure 3.6). There was no significant genotypic variability in number of flower spikes in response to light. Number of flowers and number of buds also showed large genotypic variation, both in response to temperature and to light (Figure 3.6). This was most likely due to variation in timing of consumer-ready stage between different treatments, as all plants from one genotype were harvested in one batch.

Table 3.2 Significance of genotypic variation to temperature and light. Effects on vegetative growth traits of 19 (experiment I) or 14 (experiment II) different genotypes of *Phalaenopsis* plants

	Genotype	Light x genotype	Temperature x genotype
Experiment I			
Relative growth rate	***[a]	**	***
Plant dry weight	***	***	***
Shoot dry weight	***	NS	*
Root dry weight	***	***	***
Leaf dry weight	***	NS	**
Stem dry weight	***	NS	NS
Shoot:Root ratio	***	***	***
Leaf area	***	**	**
Leaf mass area	***	**	***
Total # leaves	***	NS	*
# new leaves	***	NS	**
Experiment II			
Relative growth rate	***	NS	NS
Plant dry weight	***	NS	NS
Shoot dry weight	***	NS	***
Root dry weight	***	NS	NS
Leaf dry weight	***	NS	NS
Stem dry weight	***	***	***
Shoot:root ratio	***	***	*
Leaf area	***	NS	NS
Leaf mass area	***	*	NS
Total # leaves	***	NS	NS

[a] Interactions are: NS not significant, or significant at * $p<0.05$, ** $p<0.01$ or *** $p<0.001$

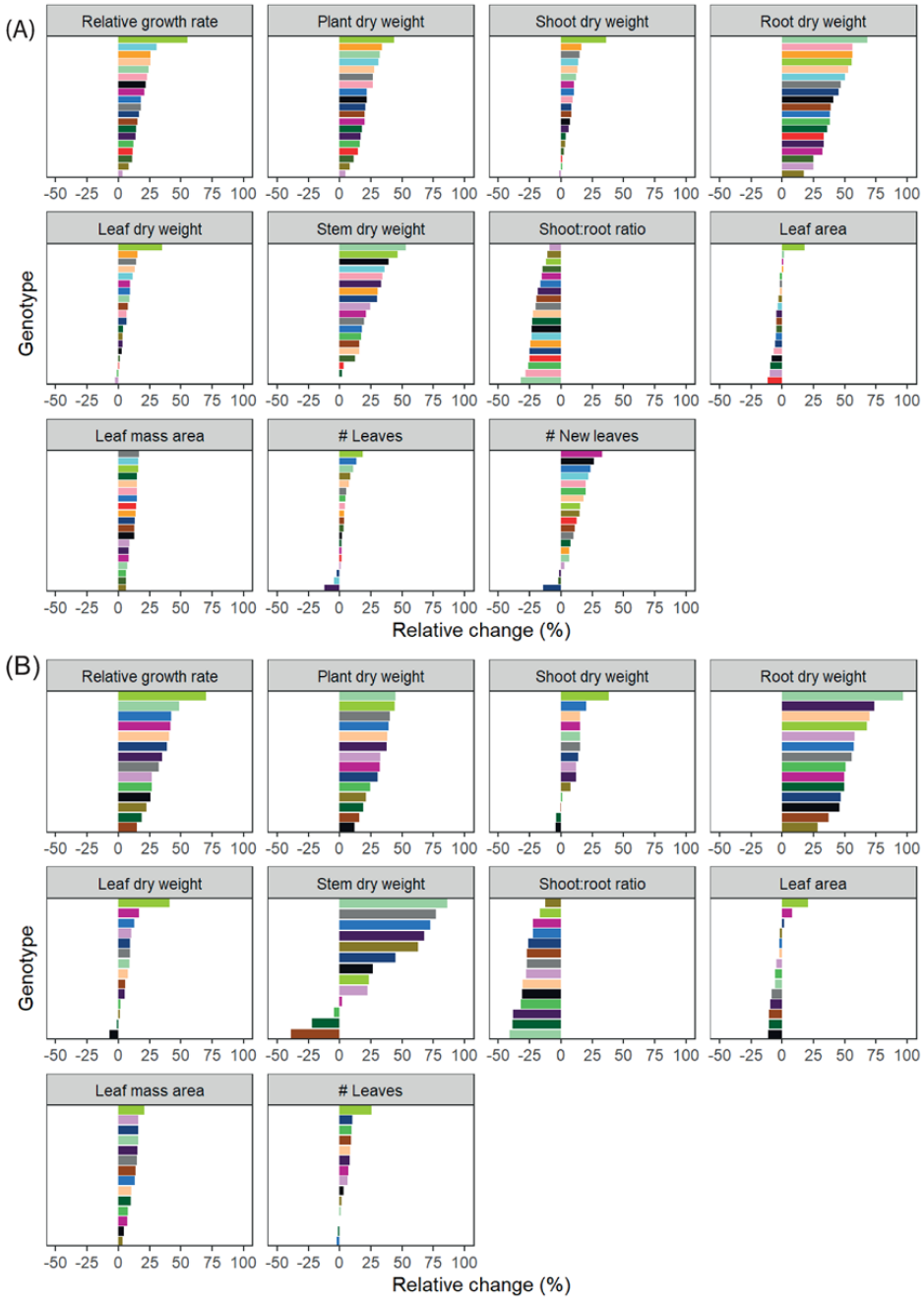


Figure 3.4 Genotypic variation in vegetative *Phalaenopsis* plants in response to light. Plants were grown in climate chambers under LED lighting for 14 hours per day at a PPFD of 60 or 140 µmol m^{-2} s^{-1}. In experiment I (A; n=3; 5-7 plants per statistical replicate per genotype), plants of 19 genotypes were grown for 19 weeks at either 27°C or 31°C. In experiment II (B; n=5; per genotype) 14 genotypes were grown for 15 weeks at either 26°C or 30°C. Data is averaged over temperature, and represents relative change per trait to light intensity (60 µmol m^{-2} s^{-1}). Similar colours are similar genotypes, also in Figure 3.5 and Figure 3.6.

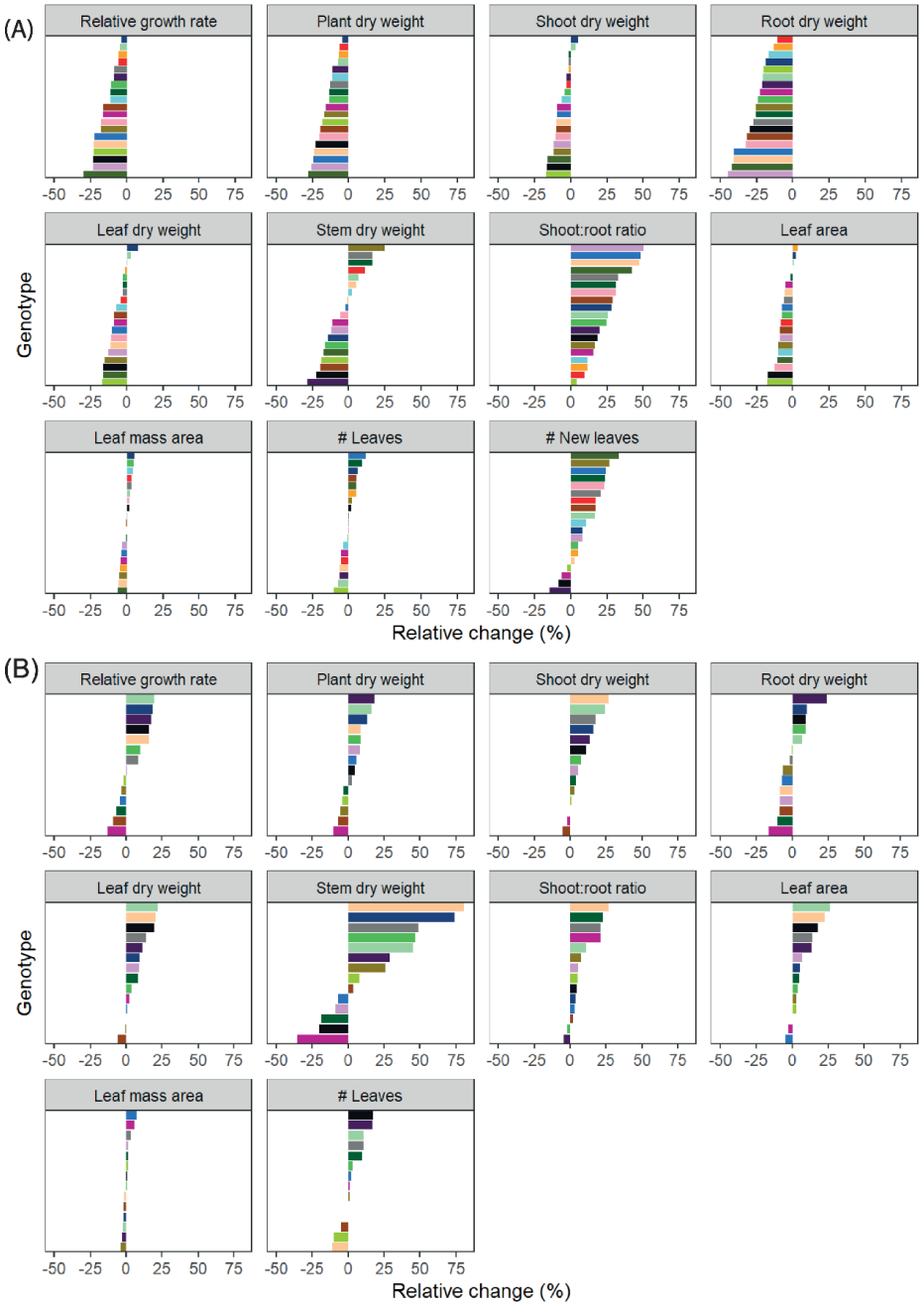


Figure 3.5 Genotypic variation in vegetative *Phalaenopsis* plants in response to temperature. Plants were grown in climate chambers under LED lighting for 14 hours per day at a PPFD of 60 or 140 µmol m^{-2} s^{-1}. In experiment I (A; n=3; 5-7 plants per statistical replicate per genotype), plants of 19 genotypes were grown for 19 weeks at either 27°C or 31°C. In experiment II (B; n=5; per genotype) 14 genotypes were grown for 15 weeks at either 26°C or 30°C. Data is averaged over light, and represents relative change per trait to temperature (27°C (A) or 26°C (B)). Similar colours are similar genotypes, also in Figure 3.4 and Figure 3.6.

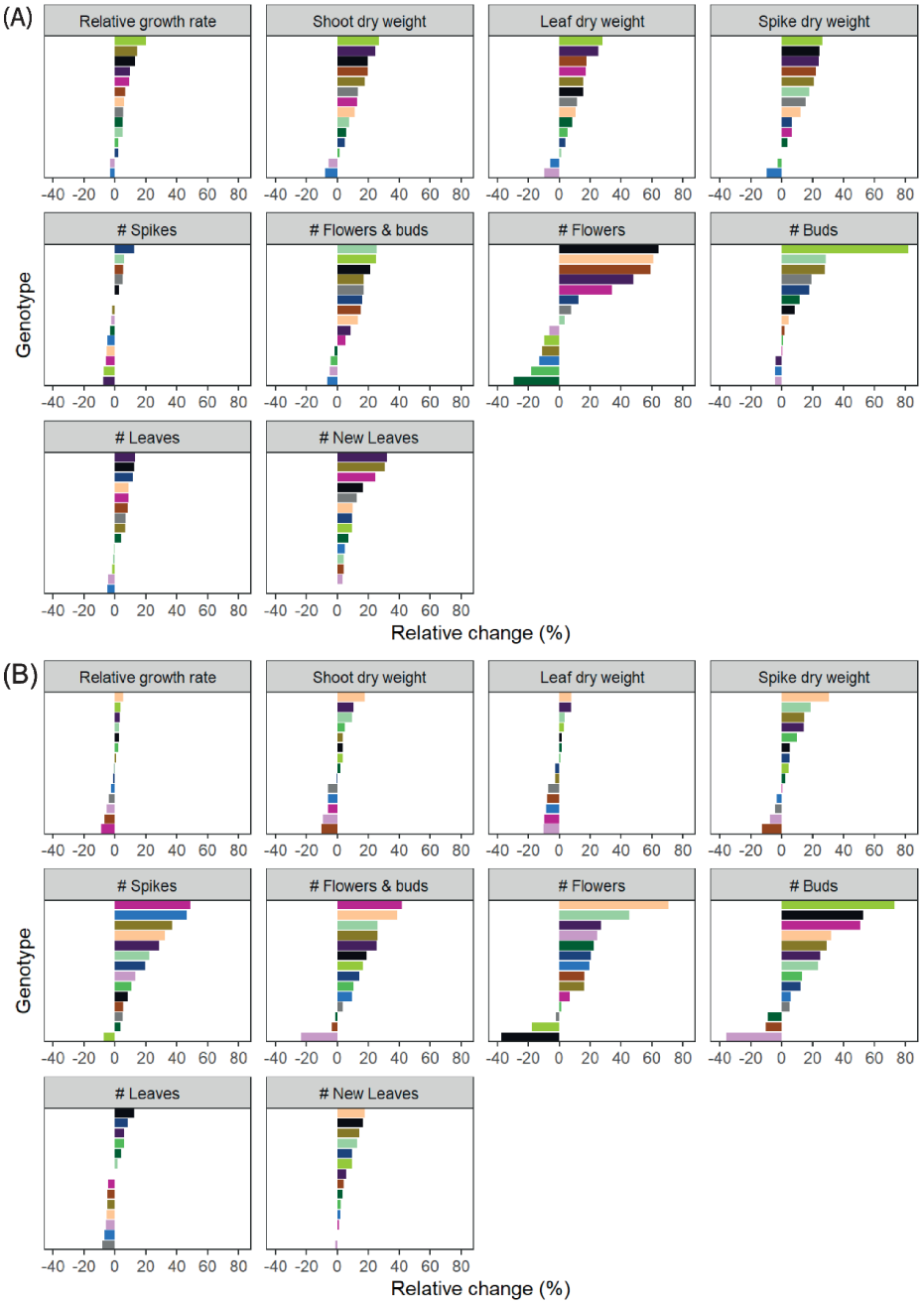


Figure 3.6 Genotypic variation in flowering *Phalaenopsis* plants of experiment II. During the vegetative phase, plants were grown in climate chambers for 15 weeks at either 26°C or 30°C and a PPFD of 60 or 140 µmol m^{-2} s^{-1} for 14 hours per day. Plants from all treatments were simultaneously moved to the greenhouse for cooling and flowering phase until auction-ready; see material and methods for details. Data is averaged either over temperature, and represents relative change per trait to light intensity (60 µmol m^{-2} s^{-1})(A) or is averaged over light, and represents relative change per trait to temperature (26°C)(B)(n=10), as applied during vegetative growth. Similar colours are similar genotypes, also in Figure 3.4 and Figure 3.5.

Table 3.2 Effect of plant type and genotypic variation and its interaction with temperature and light in flowering *Phalaenopsis*. Treatments applied in vegetative phase on after-effects during flowering of 14 different genotypes of *Phalaenopsis* plants

	Plant type	Light x plant type	Temperature x plant type	Genotype	Light x genotype	Temperature x genotype
Relative growth rate	***[a]	*	NS	***[a]	***	**
Shoot dry weight	***	NS	NS	***	***	**
Leaf dry weight	***	NS	NS	***	***	*
Flower spike dry weight	***	NS	NS	***	*	**
# flower spikes	***	NS	NS	***	NS	***
# flowers and buds	***	*	***	***	**	***
# buds	***	***	***	***	***	***
# flowers	***	NS	NS	***	***	**
Total # leaves	***	NS	NS	***	**	**
# new leaves	NS	NS	NS	***	**	NS

[a] Interactions are: NS not significant, or significant at * $p<0.05$, ** $p<0.01$ or *** $p<0.001$

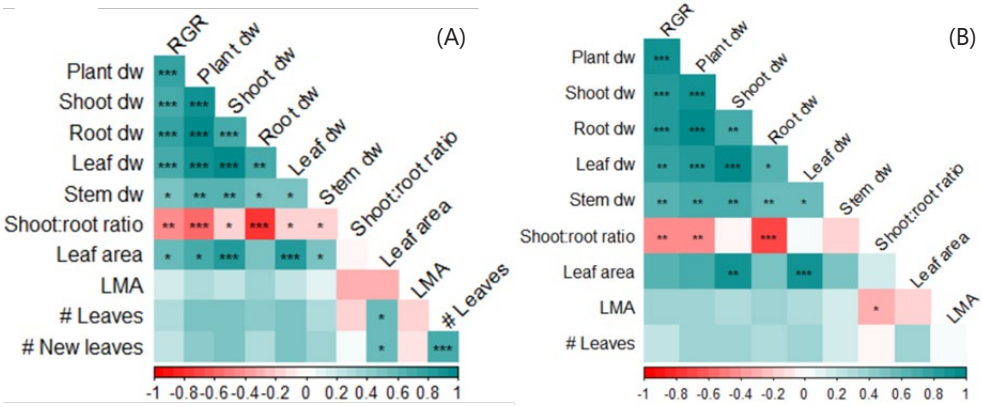


Figure 3.7 Trait correlation matrix of vegetative *Phalaenopsis* plants. Plants were grown in climate chambers under LED lighting for 14 hours per day at a PPFD of 60 or 140 µmol m^{-2} s^{-1}. In experiment I (A; n=3; 5-7 plants per statistical replicate per genotype), plants of 19 genotypes were grown for 19 weeks at either 27°C or 31°C. In experiment II (B; n=5; per genotype) 14 genotypes were grown for 15 weeks at either 26°C or 30°C. . Data is pooled over genotypes and growth treatment. Colours represent either negative (red) or positive (blue). Significant correlations are marked *,**,*** at p<0.05, p<0.01 and p<0.001, respectively. dw = dry weight, LMA = leaf mass area, RGR= relative growth rate.

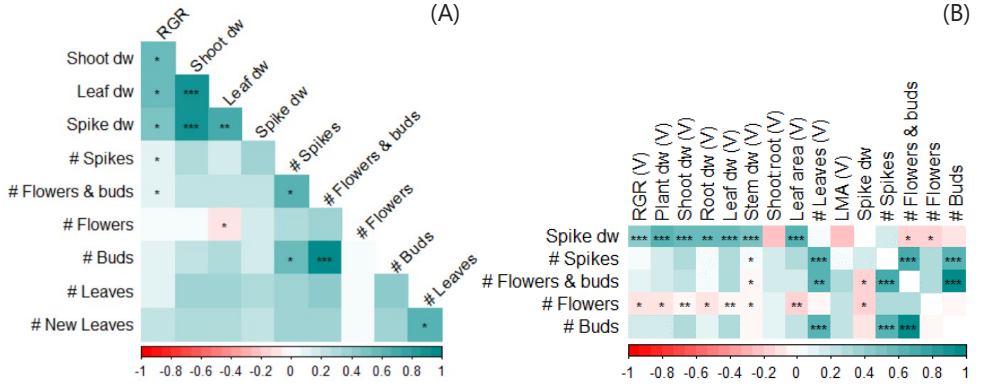


Figure 3.8 Trait correlation matrix of flowering *Phalaenopsis* plants in experiment II (A), and of vegetative (V) traits with flowering traits (B). Data is averaged over genotypes and treatments. During the vegetative phase, plants were grown in climate chambers for 23 weeks at either 26°C or 30°C and a PPFD of 60 or 140 µmol m^{-2} s^{-1} for 14 hours per day. Then, plants from all treatments were simultaneously moved to the greenhouse for flower induction and flowering phase until consumer-ready. Data is pooled over genotypes and growth treatment. Colours represent either negative (red) or positive (blue) correlations. Significant correlations are marked *,**,*** at p<0.05, p<0.01 and p<0.001, respectively. dw = dry weight, LMA = leaf mass area, RGR= relative growth rate.

3.4. Correlations between traits

Correlations between traits in both vegetative experiments were very consistent (Figure 3.7). Roots made up the largest part of plant biomass, and therefore had the strongest correlation

with total plant dry weight, as well as with shoot:root ratio. RGR was negatively correlated with shoot:root ratio, meaning that shoot:root ratio decreased as RGR increased. Root dry weight changed more with treatments, resulting in a relatively larger impact on these traits. Leaf area and leaf dry weight correlated relatively well for both experiments, although they could not be linked to the number of leaves in experiment II. This suggests that size of individual leaves reduced, as number of leaves increased. Over all treatments there was no correlation of any trait with leaf mass area, which was already apparent from previous data (Figure 3.2).

While an increase in leaf dry weight correlated with an increase in flower spike dry weight, this could not be linked to an increased flower spike number nor to an increase in flowers and buds, indicating that it was the flower spike stem weight itself that increased (Figure 3.8A). Because there were more buds than open flowers (due to time of harvest, at consumer-ready stage), the correlation between total flower potential (number of flowers and buds) was better explained by the number of buds than by the number of flowers. It was expected that more flower spikes resulted in more flowers and buds, but a higher number of flowers and buds could not be correlated to flower spike dry weight. Interestingly, neither the number of leaves, nor the number of new leaves of flowering plants was correlated to any of the flowering traits (Figure 3.8A). However, number of leaves in the vegetative phase seemed to correlate well with number of spikes, and number of flowers and buds (Figure 3.8B). Strong correlations of spike dry weight with vegetative traits were also found.

4. Discussion

In this study, we have investigated the effects of temperature and light on *Phalaenopsis* plant growth in the vegetative phase, and the after-effects of treatments applied in the vegetative phase on flowering of the plants. An increase in light intensity resulted in an increase in both plant growth and development, visible as increased biomass and plant organ development, i.e. increased number of leaves and roots. The result due to an increase in temperature seemed to depend on the temperature range that was used. The extent to which plant traits were affected by these treatments was genotype-dependent and shows the importance of genotypic variation. Flowers and buds, and number of leaves increased when light intensity and temperature increased. An increased temperature during vegetative growth also resulted in a higher number of flower spikes during flowering. Furthermore, we found that number of leaves

correlated well with important flowering traits. The implications of these results are discussed below.

4.1. Increasing light intensity stimulates growth and accelerates development of vegetative Phalaenopsis plants

Vegetative growth in *Phalaenopsis* is important and sufficient vegetative plant size is needed to develop high quality flowering plants (Runkle, 2019). Sufficient plant size is determined by the number of leaves and the plant biomass. With an increase in light intensity in the vegetative phase RGR increased, resulting in increased biomass accumulation over time for both shoot and roots (Figure 3.2 and Figure 3.3). In particular root biomass was strongly affected by light (Table 3.2), as additional dry matter was mainly allocated towards roots. Leaves were thicker (more biomass per unit leaf area) at higher light, visible as increased leaf mass area. With an increase in light more leaves were initiated, which is in line with previous results (Konow and Wang, 2001, Lee et al., 2019). In this study, increasing light intensity accelerated both growth and development.

4.2. Temperature can increase vegetative plant development but reduces plant growth in supra-optimal range

The effect of light was very similar in both vegetative experiments, whereas the effect of temperature was not. Analysing the data using identical genotypes showed that differences due to temperature treatments between the two experiments were similar to the original analysis with a full range of genotypes per experiment. This showed that differences between experiments may be due to the small difference in the range of temperature studied, and are not caused by differences in genotypes used. RGR at high temperature was significantly lower compared to low temperature treatment in experiment I. A lower RGR highly impacted root biomass accumulation (Figure 3.2). This was not the case in experiment II, where RGR and plant dry weight were not affected by temperature. In both experiments, dry matter partitioning changed with temperature, visible as decreased shoot:root ratio (Table 3.2). A previous study on *Phalaenopsis* found an increase in shoot:root ratio with increasing temperature, although

slightly lower night-time temperatures were used (Chen, 2015). It is difficult to generalize statements on the effect of temperature on shoot:root ratio, as this is very species-dependent and even varies between those sharing the same habitat (Luo et al., 2020).

Phalaenopsis employs crassulacean acid metabolism (CAM), a specialized photosynthetic pathway that temporally separates CO_2 uptake from CO_2 decarboxylation. Friemert et al. (1988) showed that temperature directly affects efflux of malate and decarboxylation of CO_2 in CAM plants, due to changes in membrane stability, which might result in CO_2 leaking out of the leaf. Furthermore, processes such as respiration, enzyme activity and stomatal movement are affected by changes in temperature, even though they might be subject to acclimation (Lüttge, 2004). Jeong et al. (2020) found that in *Phalaenopsis*, high temperature treatments, decreased relative chlorophyll content and CO_2 uptake. This might explain the lower RGR and thus lower biomass accumulation over time in our current study. It is interesting that RGR, and subsequently, plant biomass accumulation are reduced at higher temperatures if you consider the native habitat of these plants, where daytime temperatures of 31°C are not exceptional (Pridgeon, 2000).

Blanchard and Runkle (2006) showed that with a sufficiently high day temperature (>26°C), plants remain vegetative regardless of temperature during the night. While lower night temperatures have a positive effect on CO_2 uptake (Pollet et al., 2011*b*), it is unclear how this affects plant growth and development exactly. Total number of leaves did not change with temperature, but number of newly formed leaves increased in experiment I. However, leaf area decreased with an increase in temperature in experiment I (Figure 3.2). This might be due to early abscission of old leaves in experiment I. Sufficiently high temperatures accelerates plant growth and development in the vegetative phase (Runkle, 2019), but finding the optimal temperature may not be straight forward. The optimal temperature range in *Phalaenopsis* appears to be quite narrow: too low induces premature flowering in the vegetative phase, and although higher temperatures increases development of new leaves, it also leads to reduced growth and appears to accelerate aging and senescence of older leaves.

4.3. Light and temperature treatments in the vegetative phase affect flowering plant growth and development

After-effects of increased light intensity in the vegetative phase were clearly visible in flowering

plants. These plants had more leaf and flower spike dry weight, and an increased number of leaves, flowers and buds, again indicating that light affects not only growth but also plant development. Despite an increased number of leaves, the total number of flower spikes was not affected by light in the vegetative phase (Figure 3.3). Lee et al. (2019) found an increased number of flower spikes with increased light intensity, but these increased light intensities were applied during flower induction and flower outgrowth phases and not during the vegetative phase only. A different light spectrum during the cooling and flowering phase can increase number of flower spikes (Magar et al., 2019, 2020). However, to what extent light quality during vegetative growth affects flowering remains to be seen.

There was no main effect of temperature on above ground plant biomass in the flowering phase (Table 3.3), but when looking at differences between genotypes, variation occurred in both leaf and flower spike biomass (Figure 3.6). Traits related to development of organs (i.e. leaf, flower spike and flower number) increased when higher temperatures were applied in the vegetative phase. Interestingly, the effect of temperature on plant developmental rates in the vegetative phase translates to the flowering phase. It seems that the exact temperature that is applied determines what is concluded, because Jeong et al. (2020) found that an increase in temperature can lead to lower number of flower buds, and even to a reduction in number of flower spikes after high temperature stress in the vegetative phase (34°C).

Biomass production and carbohydrates play a role in flower spike outgrowth and development (Sakanishi et al., 1980, Konow and Wang, 2001). Several studies found that floral development and time to visible flower spike is positively correlated to the amount of soluble sugars in the leaves, sucrose in particular (Kataoka et al., 2004, Jeong et al., 2020, Lee et al., 2020). Sucrose levels can be increased directly via photosynthesis or exogenous sucrose application (Lee et al., 2020), but also indirectly via light spectrum (Dueck et al., 2016), or via application of plant hormones (Blanchard and Runkle, 2008), although none of these can completely substitute a low temperature treatment. From these studies it might appear that sucrose content readily available in the leaves and a continuous supply to the reproductive bud determines flower potential in the end, but sucrose alone is not the signalling factor for flower induction (Chen et al., 1994, Qin et al., 2012). *Phalaenopsis* does not have storage organs such as pseudobulbs (Christenson, 2001), but it might be that long-term storage of assimilates does

take place, which can be used later on during flowering. It remains unclear exactly how number of flower spikes and number of flowers and buds are affected by treatments applied in the vegetative phase, but there is a strong correlation between vegetative growth and flowering traits (Figure 3.8B), which highlights the importance of studying vegetative growth in *Phalaenopsis*.

4.4. Importance of genotypic variability

Within main effects of temperature and light intensity on growth and flowering in *Phalaenopsis*, genotypic variation was observed. For most traits there was an interaction of light x genotype, and/or temperature x genotype (Table 3.2, Figure 3.4 and Figure 3.5). Traits such as shoot and leaf dry weight were not significantly different on main effect level in experiment I, but they were different when genotype was considered. For instance, RGR was strongly affected by an increase in light in one genotype, but when it comes to an increase in leaves, that same genotype was performing average (Figure 3.4A). The genotype that had the largest increase in number of leaves with an increase in light intensity in experiment II in the vegetative phase (Figure 3.4B) was negatively affected by temperature (Figure 3.5B). Furthermore, in this genotype high temperature even resulted in a reduced number of flower spikes at the end of the flowering phase (Figure 3.6A). Results like these confirm the importance of including a large number of genotypes when studying Phalaenopsis growth and development (Hückstädt and Torre, 2013, Runkle, 2019). Our study showed that genotypic variation for flowering traits is large (Figure 3.6), and what is true for one genotype, might not hold for another. Hückstädt and Torre (2013) did find genotypic variability in flowering traits in response to light when two genotypes were compared. Working with a larger set of 14 genotypes showed that the number of flower spikes in most genotypes was hardly affected by light intensity in the vegetative phase. An increase in temperature in the vegetative phase increased number of flower spikes up to 50% in some genotypes, while it led to a decrease in others (Figure 3.6), which therefore might explain seemingly contradictory results in the literature (e.g. Jeong et al., 2020). Detailed information on genotypic variability can also be used as the basis for decisions on growing strategies. For instance, it might be cost-effective to invest in supplemental lighting when genotypes that respond strongly to an increase in light intensity are being cultivated, but not in those that are hardly affected.

4.5. Vegetative traits as predictor for flowering quality

In both experiments and regardless of treatment, an increase in RGR in the vegetative phase correlated with a decrease in shoot:root ratio (Figure 3.7), emphasizing the impact of root biomass on *Phalaenopsis* plant growth. This makes sense, considering that in epiphytic plants the root system is highly important, as it plays a large role in water and nutrient absorption (Zotz, 2016). However, roots are often overlooked when it comes to traits that are considered relevant in breeding and production of *Phalaenopsis*. There might be a hidden role for roots, and it is worth investigating whether root-related traits can be correlated with flower induction and flowering potential. It is generally assumed that bigger plants with more leaves result in a higher number of flower spikes, as well as a higher number of flowers per flower spike. This assumption is based on the fact that two bud primordia for flower spikes are differentiated at the base of each leaf (Rotor, 1952). Flower spikes are most likely to appear from the 3th or 4^{th} node (Sakanishi et al., 1980). While this might still be true, leaf number alone does not guarantee a certain number of flower spikes, as flower induction is also affected by other factors (Chen et al., 1994, Qin et al., 2012, Dueck et al., 2016, Runkle, 2019). Hückstädt and Torre (2013) found no relation between number of leaves and number of flower spikes on two *Phalaenopsis* genotypes studied. In our study with 14 genotypes and a combination temperature and light treatments we did find a correlation between traits measured in the vegetative phase, and traits during flowering (Figure 3.8B). Number of leaves was positively correlated with number of flower spikes, and with number of flowers and buds. Thus, increasing leaf initiation rates during the vegetative phase leads to higher flowering plant quality. Here, this was done by changing light and temperature treatments. For the orchid *Doritaenopsis*, elevated CO_2 during vegetative growth increased CO_2 uptake and leaf initiation (Yun et al., 2018). Increased CO_2 during vegetative and flowering phases in *Phalaenopsis* resulted in more branching and more flowers, indicating that flowering quality increased (Trouwborst et al., 2016). Linking data of vegetative plants grown at different environmental conditions with flowering characteristics later on, can assist in the selection of new cultivars during breeding, as it can be used as an early predictor for flowering capacity and quality.

5. Conclusion

We studied the interaction between temperature and light on growth and development of *Phalaenopsis*, so that leaf initiation rate and dry matter production would be optimized. These traits in particular were considered important in the vegetative phase, as they would result in higher quality flowering plants. This study has led to several new insights. 1) Increasing light intensity accelerates both plant growth and development in *Phalaenopsis*. 2) Increasing temperature can accelerate plant development, but can quickly lead to reduced growth when supra-optimal. 3) Genotypic variation in the response to temperature and light is large in *Phalaenopsis*, especially in traits related to flowering. Therefore, sufficient genotypic variation in studies is important and care is needed when generalising results. 4) Growth in the vegetative phase can be linked to flowering traits. The positive correlation between number of leaves during the vegetative phase and number of flower spikes can be used to predict flowering capacity and quality of the final product.

6. Acknowledgements

We would like to thank Anthura and Joke Oosterkamp (Wageningen University and Research) for all help with experimental work and conducting measurements. We would like to thank Gerrit Gort (Biometris, Wageningen University and Research) for helping with statistical analysis.

Supplemental information S3

S3.1 LED light spectrum in climate chamber

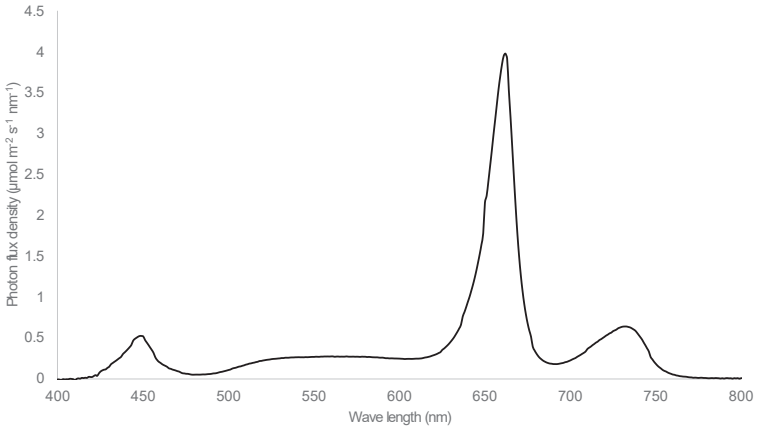


Figure S3.1 Representative spectral distribution in climate chamber. PPFD of 140 µmol m^{-2} s^{-1} and additional far-red of 23 µmol m^{-2} s^{-1}. Spectrum was composed of red, white and far-red LED modules (Philips GreenPower LED production module Deep Red/White and GreenPower LED research module Far Red).

S3.2 Plant watering nutrient solution

Plants were watered with a nutrient solution (EC 1.2 mS cm^{-1} and pH of 5.7) composed of the following:

Table S3.1 Composition of nutrient solution

Ion		Concentration
Macro	$N\text{-}NO_3$	5.6
(mmol l^{-1})	$N\text{-}NH_4$	1.2
	CH_4N_2O	7.4
	P	1.4
	K	3.5
	Ca	1.3
	Mg	0.7
	SO_4	0.7
Micro	Fe	36.7
(µmol l^{-1})	Mn	8.2
	Zn	1.9
	B	15.1
	Cu	1.9
	Mo	0.8

S3.3 Phenotypic description of Phalaenopsis genotypes

Genotype (coded as UPO#) 1,2 and 4-20 were used in experiment I, and genotypes 1-14 were used in experiment II (Table S3.2)

Table S3.2 Phenotypic description of Phalaenopsis genotypes.

Genotype Number (UPO)	Color	Picture	Specific characteristics of each genotype	Cultivation time (weeks)	Leaf initiation (leaf week^{-1})	Number of flower spikes	Height of flowering plant (cm)
1	White		Reference genotype	66.37	0.110	2.06	61.83
2	White		Highest leaf initiation rate and highest number of flower spikes	64.14	0.144	3.08	58.57
3	White with pink stripe		Low number of flower spikes compared to number of leaves (opposite of genotype 7). Big difference in summer and winter growth	69.67	0.094	1.78	63.33

Table S3.2, continued

4	White, red lip		Similar winter and summer growth rate and development	67.58	0.098	1.90	66.59
5	Purple		Similar winter and summer growth rate and development	68.86	0.093	1.80	65.00
6	Yellow		Slow grower, dark green leaves	73.55	0.086	1.88	54.47
7	White		Fast grower, high number of flower spikes (reduced cooling phase). High leaf initiation rate, but also sensitive to bud abortion	66.31	0.121	2.89	60.97

Table S3.2, continued

8	Red		Sensitive to light, slow growth rate of young plants. Sensitive to leaf damage in winter	69.49	0.099	1.94	64.81
9	Red-copper		Sensitive to premature flowering. Leaves have hint of red, anthocyans	68.24	0.101	1.80	62.50
10	Pink		Few, large leaves. High number of flower spikes. Highly responsive to changes in light quality	67.38	0.106	2.09	68.42
11	Purple		Multiflora. Flower spikes show high amount of branching.	68.41	0.124	2.55	44.64

Table S3.2, continued

12	White		Multiflora. Small leaves with the tendency to variegate. Relatively large roots	67.91	0.102	2.22	55.36
13	Lilac		Multiflora. Variegated leaves, botanical background	*	*	*	*
14	White		Multiflora	58.86	0.136	2.60	50.00
15	White with pink stripe		Sensitive to leaf damage in cooling phase	67.98	0.093	1.96	64.00

Table S3.2, continued

16	Pink		Multiflora	69.46	0.127	2.43	50.00
17	White		Plants have average flower size	65.54	0.149	2.23	50.50
18	Pink		Very robust and though leaves, with a lighter shade of green. Opposite of genotype 4 and 6, which have leaves that are darker coloured and the tendency to produce antocyanins.	71.56	0.085	1.92	43.85
19	Lilac		Often variegated leaves	68.77	0.114	1.94	52.08

Table S3.2, continued

20	Yellow		Low leaf initiation rate, resulting in low number of leaves	72.38	0.085	1.97	61.25
			Average	68.43	0.104	2.13	58.98

S3.4 Genotypic variation

S3.4.1 Similarity matrix

Genotypic similarity in this study was determined based on the variety tracer method, developed by NAKtuinbouw (Roelofarendsveen, The Netherlands) to identify plant *Phalaenopsis* varieties. This is done based on the presence or absence of different alleles for 8 SSR markers (Ben-Ari and Lavi, 2012)[H. Teunissen, personal communication]. Alleles were dominantly scored (based on the presence/absence of polymorphic DNA fragments). In case of doubt during scoring bands were scored as 'uncertain', which means that the allele is considered as neither present nor absent. These scores were ignored in the statistical genetic analysis. In order to investigate the genetic relationship of samples, a data set of absent/present marker scores was generated in a score table. For a simplified representation of these results, techniques such as clustering and ordination analyses are generally employed. The predecessor of these analyses is the construction of a similarity (or distance) matrix. The genetic similarity is the proportion of molecular markers (values varying from 0 to 1 or 100%) that are shared between the two samples being compared. For construction of the complete matrix all samples are compared to all samples. Several different coefficients have been proposed (Sneath and Sokal, 1973) but for the use of molecular markers similarity coefficients specific for binary variables (presence/absence) are suggested. The similarity matrix was calculated applying the most commonly used 'Jaccard' (*a/n-d*) coefficient (Sneath and Sokal, 1973, Vierling and Nguyen, 1992, Zhang et al., 2012). BioNumerics' software (Applied Maths, Sint-Martens-Latem, België) was used to produce a similarity matrix.

The Jaccard coefficient is the international standard for similarity calculations based on binary data. a, b, c, d, m, n, and u are defined as follows for a two-way frequency table comparing two samples i and j. + present markers; - absent markers.

$m=a+d$ (number of matched)

$u=b+c$ (number of un-matched)

$n=u+m$ (total sample size)

	j		
i		+	-
	+	a	b
	-	c	d

Jaccard = $a/(n-d)$

Table S3.2 Genotypic similarity between *Phalaenopsis* genotypes (UPO#) based on the presence or absence of 8 SSR markers, expressed as percentage. Different numbers represent different genotypes

UPO	1																		
1	100	2																	
2	100	100	3																
3	30	30	100	4															
4	30	30	27	100	5														
5	33	33	22	13	100	6													
6	26	26	19	10	13	100	7												
7	19	19	17	24	10	11	100	8											
8	32	32	25	11	22	20	8	100	9										
9	24	24	29	24	33	30	13	27	100	10									
10	32	32	21	24	23	13	22	21	16	100	11								
11	20	20	30	9	16	21	10	31	28	23	100	12							
12	38	38	20	28	18	24	21	30	26	36	17	100	13						
13	9	9	11	6	9	0	10	10	6	12	12	10	100	14					
14	15	15	10	12	24	4	18	8	7	11	10	8	11	100	15				
15	24	24	15	25	13	13	10	22	19	28	12	22	6	7	100	16			
16	33	33	31	25	23	26	23	32	29	32	33	33	19	13	14	100	17		
17	38	38	19	27	21	10	30	7	21	21	13	19	6	12	13	16	100	18	
18	33	33	30	17	29	32	7	27	38	23	32	27	6	11	20	28	17	100	19
19	16	16	21	16	16	9	22	13	18	27	9	21	12	15	19	14	25	19	100	20
20	52	52	37	23	27	29	17	30	30	22	26	35	9	14	23	27	20	27	18	100

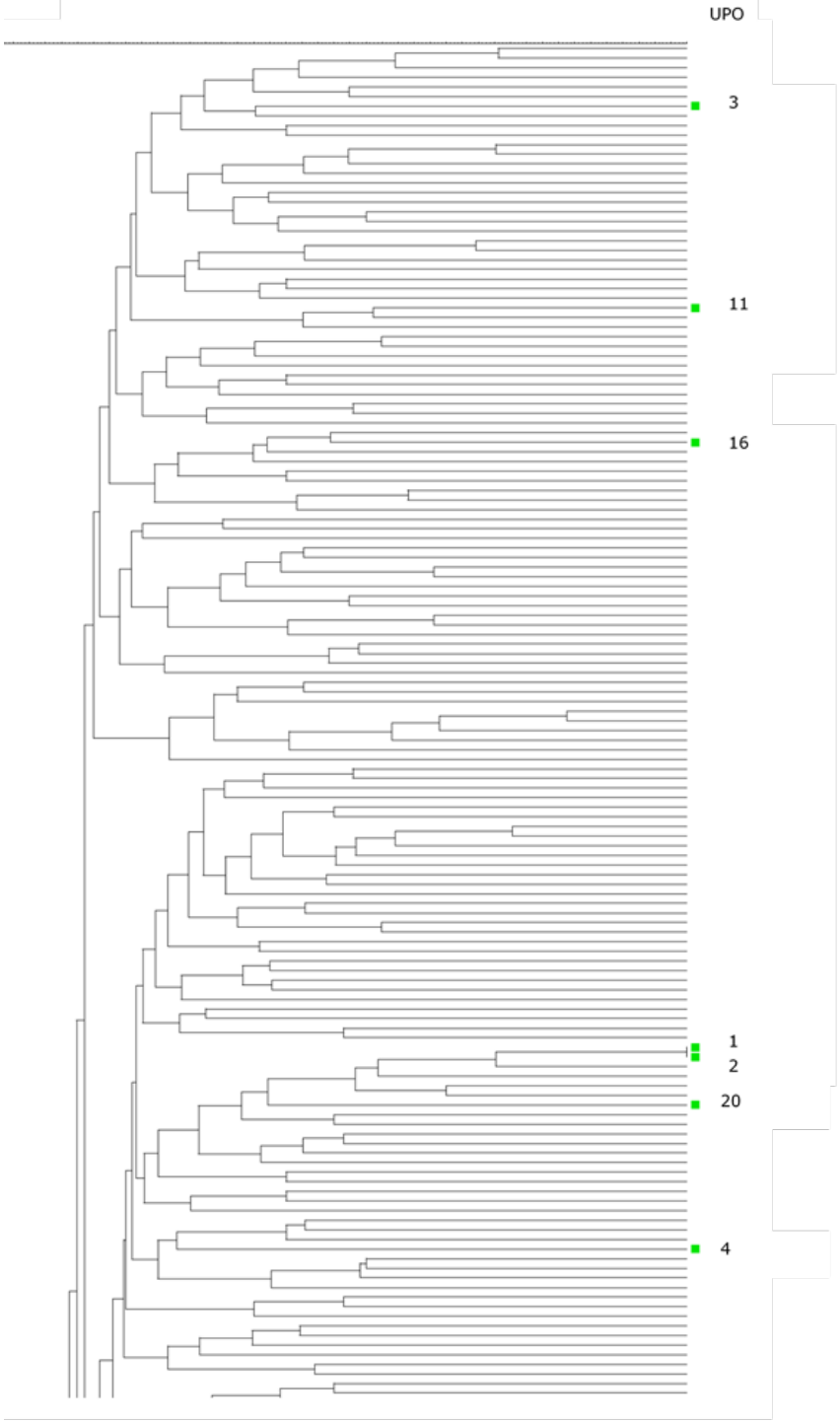
S3.4.2 Dendrogram

In order to further display genotypic variation, a dendrogram was created that shows the variation of the genotypes used in this study relative to the complete genotypic pool of the breeder, from which plants were acquired (Figure S3.2). To visualize the relationship between the samples a dendrogram was generated using UPGMA parameters (Unweighted Pair-Group Method, Arithmetic average). This kind of algorithms find successive clusters using previously established clusters. Two steps are performed repeatedly: 1. find and merge the two best matches and 2. update the similarity matrix by averaging the scores.

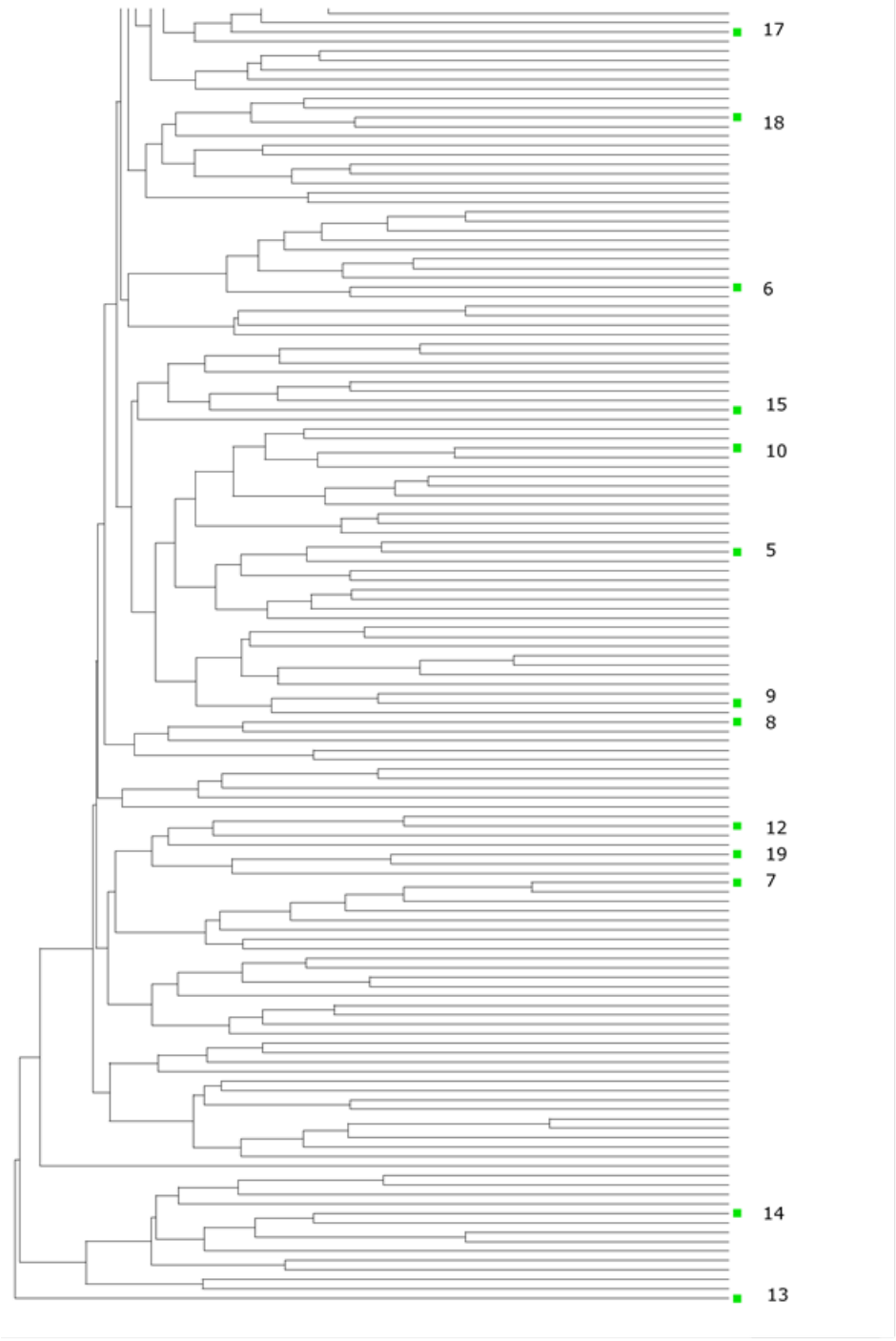
S3.4.3 Principal component analysis

Principal component analysis (PCA) was done on 278 *Phalaenopsis* samples in three dimensions based on 166 alleles. The first dimension explains 6.3% (x- axis), the second 5.5% (y- axis) and the third principal co-ordinate explains 4.7% (z- axis) of the total variation (Figure S3.3) Coloured group corresponds with the coloured group in the dendrogram.

Figure S3.2 Dendrogram showing 278 examined *Phalaenopsis* samples based on the score of 166 alleles using the 'Jaccard' similarity coefficient and UPGMA analysis. On the horizontal axis, the similarity is given.



Continuation of dendrogram



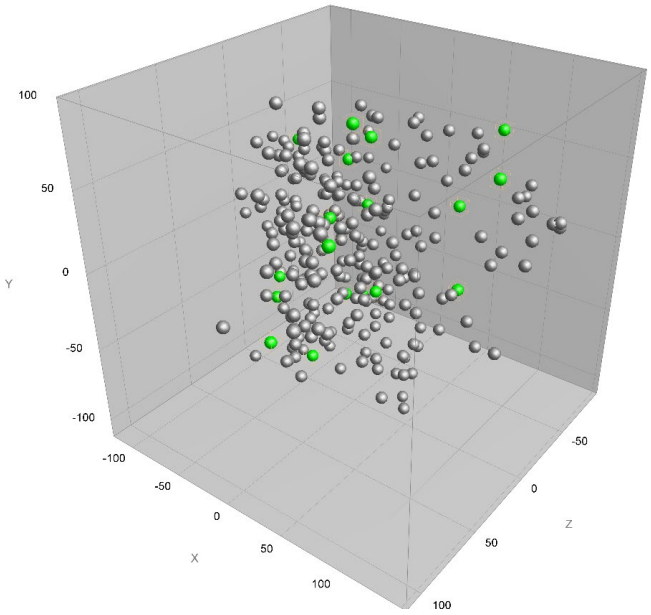


Figure S3.3 Principal component analysis of 278 *Phalaenopsis* samples in three dimensions based on 166 alleles.

S3.5 Genotypic variation in flowering Phalaenopsis based on plant type

Breeding in *Phalaenopsis* focusses on creating either smaller plants with a high number of small flowers, or regular sized plants with fewer, but larger flowers and buds; referred to as Multiflora (light grey) and Grandiflora (dark grey), respectively. During the vegetative phase, plants were grown in climate chambers for 15 weeks at either 26°C or 30°C and a PPFD of 60 or 140 µmol m^{-2} s^{-1} for 14 hours per day. Plants from all treatments were simultaneously moved to the greenhouse for cooling and flowering phase until auction-ready; see section 2.2, experiment II for details.

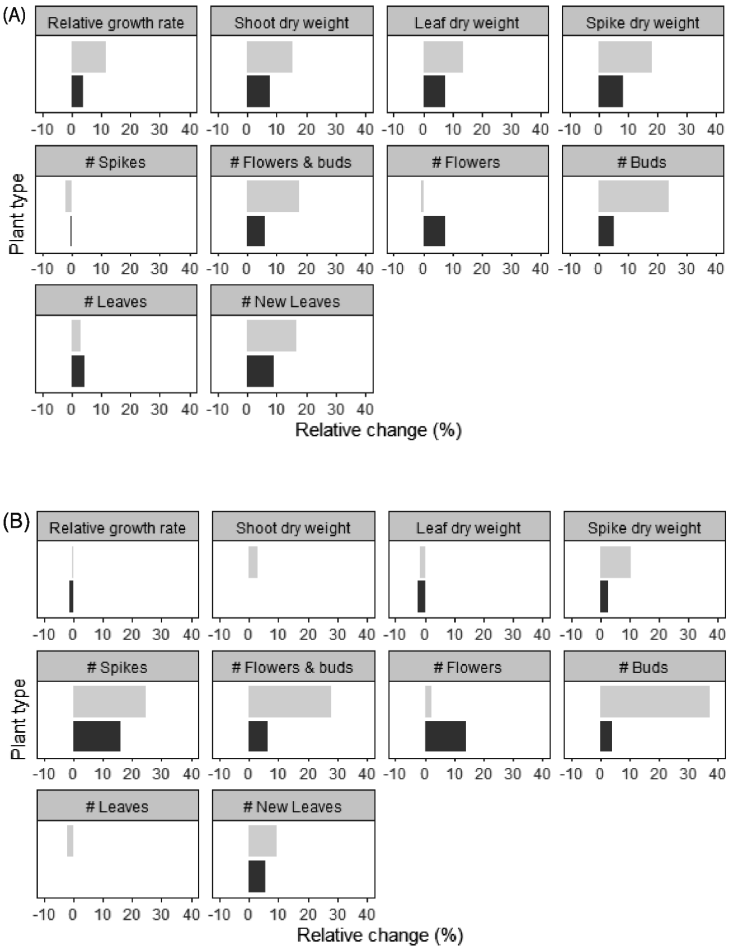


Figure S3.4 Average relative changes per plant type, Multiflora (light grey) and Grandiflora (dark grey) per trait to either vegetative reference light intensity (60 µmol m^{-2} s^{-1})(A) temperature (26°C)(B).

Chapter 4

Linking diel measurements of gas exchange and assimilates to plant growth in the CAM-plant Phalaenopsis

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Abstract

Phalaenopsis orchids are epiphytes that engage CAM photosynthesis, a specialized photosynthetic pathway that temporally separates CO_2 uptake during the night from carboxylation during the day. We aimed to find what causes differences in growth and development in *Phalaenopsis* genotypes in response to the environment. We exposed vegetative plants of two genotypes (UPO4 and UPO6) to different temperature (27 or 31°C) and light intensity (60 or 140 µmol m^{-2} s^{-1}) treatments for 28 weeks. Measurements of gas exchange rates and carbohydrate analysis within a diel cycle were combined with measurements on plant growth and development. Plants from UPO4 were larger than plants from UPO6, and both were larger at 27°C compared to 31°C. Light intensity did not affect biomass accumulation. Significant CO_2 leakage out of the leaf occurred at 31°C, which likely explained reduced growth compared to 27°C. Net cumulative diel CO_2 uptake expressed per plant correlated with vegetative biomass accumulation. Hierarchical clustering revealed that temperature was the dominating determinant of variation observed, followed by light intensity. Using PCA we showed that the two genotypes varied in how they acclimate to environmental conditions in order achieve their final biomass. This variation was visible in parameters related to CAM photosynthesis (visible in CO_2 uptake and transpiration), assimilate allocation among carbon pools (visible in allocation of sugars and starch), and in the allocation of biomass towards different plant organs (visible in number and thickness of leaves, and in roots). These results can support breeding by selection of specific traits based on physiology.

Keywords: Carbon budget, crassulacean acid metabolism (CAM), gas exchange, Phalaenopsis, plant growth

1. Introduction

Crassulacean acid metabolism (CAM) is a specialized photosynthetic pathway with temporal separation of CO_2 uptake, storage, release and assimilation. This form of carbon concentrating mechanism results in an increased water-use efficiency (WUE)(Yang et al., 2015). Photosynthesis in CAM plants is best described by four phases within a diel cycle (Osmond, 1978). The main phases are phase I and III. Phase I takes place during the night, when stomata are fully open, and CO_2 uptake is catalysed by phosphoenolpyruvate carboxylase (PEPC) and subsequently stored in the vacuole as a C_4 acid (Chen et al., 2008). In phase III, which occurs during the day, stomata are fully closed, and previously stored C_4 acid is decarboxylated, and subsequently released CO_2 is assimilated by Rubisco in the Calvin-Benson cycle. Transitional phase II starts at dawn. Simultaneous activity of PEPC and Rubisco may cause an early morning burst of CO_2 assimilation in this phase (Osmond et al., 1996, Lüttge, 2001, Borland and Dodd, 2002). End of day re-opening of stomata marks the start of transitional phase IV, mainly due to the depletion of C_4 acid and drawdown of available internal CO_2 (C_i). The duration of transitional phases II and IV, where stomatal closure is delayed in the morning, or might remain closed longer at the end of the day, differs between species and depends on the environment (Lüttge, 2001, Von Caemmerer and Griffiths, 2009). Additional restrictions to metabolic control may be imposed by the circadian clock (Hartwell, 2006). In order to fix CO_2 during phase I, CAM plants store additional carbohydrates during the day (sugar or starch, depending on plant species). These carbohydrates form the basis for phoshoenolpyruvate (PEP), the substrate required for nocturnal CO_2 fixation. The additional carbohydrate sink of PEP requires CAM plants to carefully balance their carbohydrate pools between nocturnal substrate formation and export for growth. This balance depends largely on environmental conditions. Contrary to C_3 and C_4 species, the highly plastic and adaptive nature of CAM plants and their carbon balance has not yet been studied extensively.

Common CAM crops include *Phalaenopsis* orchids, epiphytes that originate from tropical rainforests in south-east Asia. Epiphytes have no direct access to soil and are constrained by the availability of water and nutrients. To overcome these limitations, *Phalaenopsis* orchids engage CAM (Gilman and Edwards, 2020). *Phalaenopsis* plants are grown in greenhouses, to ensure year-round, high quality production (Anthura, 2017). In *Phalaenopsis*, strong correlations have been found between vegetative growth traits, such as number of

leaves, relative growth rate and plant biomass, and quality of the flowering plant (Chapter 3). Plants that had more biomass at the end of the vegetative phase showed increased spike dry weight (Figure 3.8B), a trait that represents flowering quality. Interestingly, optimal nocturnal CO_2 uptake per m^2 leaf area was not linked to highest biomass accumulation in *Phalaenopsis* (Pollet et al., 2011). Studying vegetative growth and optimizing the response to temperature and light during vegetative growth phase can shorten time to flowering, or result in higher commercial plant quality (i.e. a higher number of flower spikes and higher number of flowers). The temperature for vegetative growth of *Phalaenopsis* should be sufficiently high to suppress premature flowering, while supra-optimal temperatures can negatively affect leaf initiation rates (Figure 3.5). Several studies showed a positive correlation between growth and light intensity (Konow and Wang, 2001, Dueck et al., 2011, Lee et al., 2019). Interactions between light and temperature in *Phalaenopsis* have been reported, e.g. Lootens and Heursel (1998) found that saturating light levels increased with an increase in temperature. While some evidence for interaction of temperature and light exists, the effects of short-term processes, such as CO_2 uptake and carbohydrate content on long-term growth and development are largely unexplored.

There is some evidence of genotypic variation in *Phalaenopsis* in response to temperature and light. Initially, Hückstädt and Torre (2013) compared two *Phalaenopsis* genotypes and found that an increase in light intensity during the vegetative phase increased leaf soluble carbohydrates in one genotype, while the other genotype was not affected. More recently, a study with 19 *Phalaenopsis* genotypes showed that, despite large variation in genotypic response to temperature and light, a correlation between the number of leaves and flowering, and plant biomass exists (Chapter 3). However, the underlying variation in physiology has not been studied. In CAM plants, biomass accumulation and yield has not been studied extensively, because CAM is generally considered an unfavourable trait in agriculture (Davis et al., 2019). Models can be used to predict biomass in relation to temperature and light and these have been developed for several horticultural species (e.g. for tomato, see Heuvelink 1999; for rose, see Kim and Lieth 2003, Zhang et al. 2020). However, these models are unsuitable for *Phalaenopsis*, because of their CAM physiotype. Therefore, in order to study the biomass accumulation in relation to temperature, light and its underlying physiology of the CAM species *Phalaenopsis*, we will make use of the CAM framework proposed earlier (Chapter 2). This

framework provides direction on how to combine data obtained at different biological and temporal scales, and aims to serve as the foundation for a quantitative model of *Phalaenopsis*.

With this study we aimed to further unravel the physiology of CAM in *Phalaenopsis*, by studying differences in vegetative growth in response to combinations of environmental factors (temperature and light) between two genotypes. Therefore, we combined short-term measurements of photosynthetic gas exchange with metabolite analysis (malate, citrate, sucrose, fructose, glucose and starch), and linked these results to long-term measurements of growth and development. By doing so, we were able to study correlations between CAM photosynthesis, metabolism, and growth and development in *Phalaenopsis*. These insights can help in the development of a quantitative growth model of *Phalaenopsis*, and can be used to identify key parameters in order to select for enhanced plant biomass and quality in breeding of *Phalaenopsis*.

2. Material and Methods

2.1. Plant material

Vegetative *Phalaenopsis* plants of two genotypes (named UPO4 and UPO6) were grown in a Venlo type greenhouse (Bleiswijk, The Netherlands) in 12 cm transparent pots filled with coconut bark. Genotypes used in this study were provided by breeding company Anthura (Bleiswijk, The Netherlands)(see Supplemental information S3.3 and S3.4 for detailed information on phenotypes and genetic similarity between genotypes that were used here). At the start of the experiment plants were transferred to two climate chambers, set to either 27°C or 31°C. Per climate chamber, six compartments were created for the light treatments. Plants were illuminated at a PPFD of either 60 or 140 µmol m^{-2} s^{-1} and additional far-red of 10 or 23 µmol m^{-2} s^{-1}, respectively (see S3.1 for a representative spectrum). Light treatments were applied for 14 hours per day, using red/white, and far-red LED modules (Philips LED production module deep red/white and GreenPower LED research module Far Red; Signify, Eindhoven, The Netherlands). The ratio of red (600-700 nm) to far-red light (700-800 nm) was approximately 1.2, and the estimated photostationary state (PSS) was 0.83 (Sager et al., 1988). Daily light integral was 3 or 7 mol m^{-2} day^{-1}. Vapour-pressure deficit of the air was set at 1 kPa for all treatments. Plants were watered every 5 days with a nutrient solution with an EC 1.2 mS cm^{-1}

and pH of 5.7. For composition of the nutrient solution, see supplemental information S3.2. CO_2 concentration of the air was kept at 600 ppm. The youngest fully grown leaf was marked with a peg. Plants were considered separate, since there was no mutual shading at the plant density of 12 plants m^{-2}.

2.2. Biomass and development

Plants were destructively harvested after 28 weeks (5 plants per compartment). Numbers of old and new leaves were counted. After removing all coconut bark substrate, roots were cut off as close to the base as possible. Leaves were carefully removed so that the stem remained intact, and leaf area was determined (Li-3100, Li-Cor Inc., Lincoln, USA). Dry weights of roots, leaves and stem were determined after drying plant material for at least 48 hours at 80°C. Specific leaf area (SLA, in m^2 kg^{-1}) was calculated from leaf area of the plant and leaf dry weight.

2.3. Gas exchange

Starting in week 26 after start of the experiment, gas exchange measurements were done on the youngest mature leaf of one plant per compartment using LI-6400XT (n=3) (Li-Cor Inc., Lincoln, USA) with a transparent chamber (6 cm^2). Measurements were done at a flow rate of 100 µmol m^{-2} s^{-1} with a block-controlled temperature of either 27°C or 31°C. In order to achieve sufficient air mixing and to dampen fluctuations in concentration, a buffer volume of at least 25L with a fan was connected to the air inlet. The order of the measurements was randomized over treatments and genotypes, and always started during phase III when stomata were closed. Data were logged every 5 minutes taking an average of 20 seconds.

2.4. Sampling and analysis of assimilates

Leaf discs of the youngest mature leaf of one plant per compartment (n=3) were taken for biochemical analysis at 7 time points throughout a diel cycle, in week 27 after start of the experiment. Sampling was done from the same plant, starting at the top of the leaf. Per replicate, a pooled sample of leaf material with a total of 3 cm^2 was collected from leaves of three plants, weighed and placed in liquid nitrogen and stored at -80 °C. Leaf discs were freeze-

dried and ground to powder with two metal pellets (3mm) in the Eppendorf for one minute using a ball mill. Samples were stored in a desiccator until further processing. All assimilates were quantified using High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD; Dionex ICS5000, Thermo Fisher Inc.), according to the method described by van Tongerlo et al. (2020, chapter 5 of this thesis). The total accumulation of malate, citrate, sucrose, fructose, glucose and starch was calculated in mmol m^{-2} using leaf mass area of leaf discs. From this data, the accumulation or breakdown during day and night was calculated per metabolite and is expressed as Δ.

2.5. Statistical setup and analysis

Two climate chambers were used, corresponding with temperature treatments. We assumed temperature, alongside light intensity, was replicated independently, which may have underestimated random variance. Six separate compartments were created per climate chamber, allowing each light treatment to be replicated three times. Within these compartments, plants from the two genotypes were randomized. All data were analysed using R version 4.0.2 (R Core Team, 2019). Principal component analysis (PCA) was conducted per genotype to a parameter x treatment (P x T) matrix with standardized data transformation (see Table 4.1 for a full list of parameters that were used in this matrix). To assure independence of variables, we created a P x T correlation matrix per genotype. Correlations were tested using Pearson's correlation coefficient and checked for significance at $p<0.05$ (package corrplot). Hierarchical clustering was conducted, using Ward's minimum variance method, based on squared Euclidian distance (Ward, 1963) on the P x T matrix of standardized means. Furthermore, effects of light and temperature treatments were analysed using linear mixed-effect models (package lme4) at $p<0.05$. Data were checked for homogeneity and normality, and transformed where needed using square root transformation (leaf area) or log transformation (root dry weight, CO_2 uptake phase II, duration of phase II, transpiration of phase II).

3. Results

Genotypes responded differently in terms of growth in the vegetative phase to changes in environment. Plants of both genotypes grown at 31°C had a lower plant dry weight than plants grown at 27°C, but this effect was larger for UPO4 than for UPO6 (Figure 4.1A,B). Diel CO_2 uptake was higher at the higher light intensity in both genotypes (Figure 4.1C,D), and higher at 27°C compared to 31°C. The increased CO_2 uptake at 140 µmol m^{-2} s^{-1} was mainly caused by an increased CO_2 uptake in phase I (Figure 4.2), although CO_2 uptake in phase I was higher at 27°C compared to 31°C. At 31°C, there was CO_2 leakage out of the leaf during phase III in both species. At 31°C and 60 µmol m^{-2} s^{-1}, 44% and 49% of CO_2 that was taken up during phases I, II and IV leaked out during phase III in UPO4 and UPO6, respectively. Initially, there appeared to be only a weak correlation between CO_2 uptake per m^2 leaf and plant dry weight (r^2=0.42 and r^2=0.16 for UPO 4 and 6, respectively). However, when expressed on a per-plant basis, diel CO_2 uptake correlated better with plant dry weight (r^2=0.79 and r^2=0.45 for UPO 4

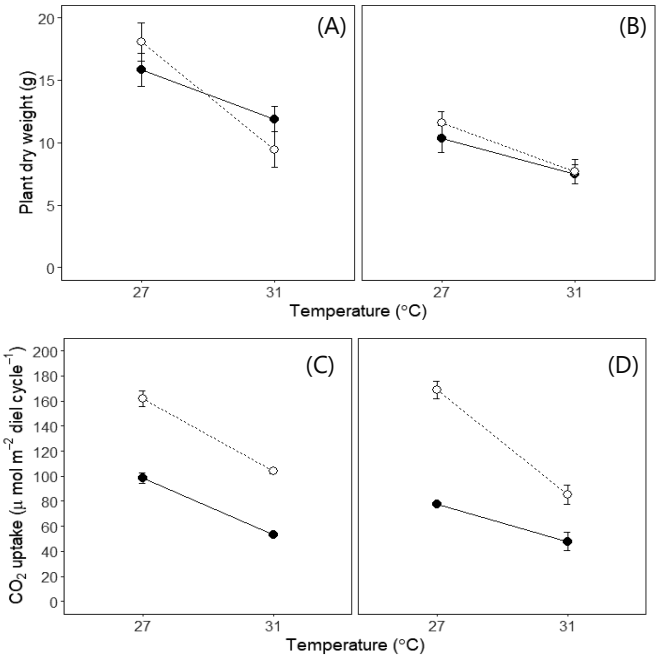

Figure 4.1 Plant dry weight (A,B) and diel cumulative CO_2 uptake (C,D) of vegetative *Phalaenopsis* plants of two genotypes, UPO4 (A) and UPO6 (B) grown for 28 weeks at different combinations of temperature (27°C or 31°C) and light (PPFD of 60 µmol m^{-2} s^{-1} (closed circles) or 140 µmol m^{-2} s^{-1}(open circles)). Data represent averages (n=3 with 5 replicate plants each) ±SEM error bars.

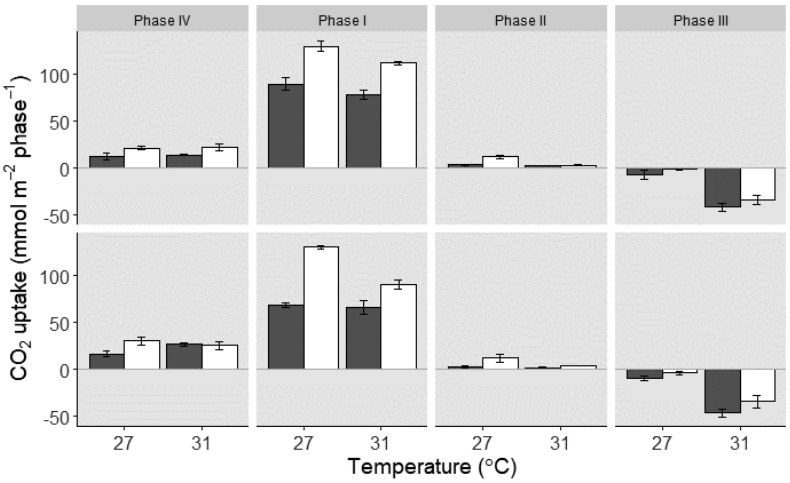

Figure 4.2 Cumulative CO_2 uptake per CAM-phase of vegetative *Phalaenopsis* plants of two genotypes, UPO4 (top) and UPO6 (bottom) grown at different treatment combinations of temperature (27°C or 31°C) and light (PPFD of 60 µmol m^{-2} s^{-1}(grey bars) or 140 µmol m^{-2} s^{-1}(white bars)). Data represent averages (n=3) ±SEM error bars. CAM-phases expressed according to Osmond (1978).

and 6, respectively)(Figure 4.3), although this relationship varied with genotype and in particular, with temperature. UPO4 responded more strongly to different environmental conditions than UPO 6, both in the amount of CO_2 that is taken up, as in how CO_2 uptake correlated with plant dry weight (Figure 4.3). While plant dry weight of UPO4 at 31°C was lower at 140 µmol m^{-2} s^{-1} than at 60 µmol m^{-2} s^{-1}, these plants took up more CO_2 over a diel cycle (Figure 4.3). In UPO6, CO_2 uptake at 31°C differed between light intensities, but this did not result in a difference in plant dry weight.

These results indicated that the two genotypes might use different strategies in the process from CO_2 uptake to biomass, in order to deal with different environmental conditions. The effect of environmental conditions was explored using PCA for each genotype. PCA can be used to reduce the dimensions of a multi-dimensional dataset to find the components that explain the most variance in a higher dimensional data cloud. Per genotype, 30 measured and derived parameters from four treatments were included in the PCA. The first two principal components (PCs) cumulatively explained 90% and 86% of the variance for UPO4 and UPO6, respectively. The parameters that accounted for the most variation with each PC varied between genotypes (Table 4.1, Supplemental information S4.1). Parameters that were most strongly correlated with PC1 in UPO4 were transpiration in phase III and $\Delta_{sucrose}$ during the

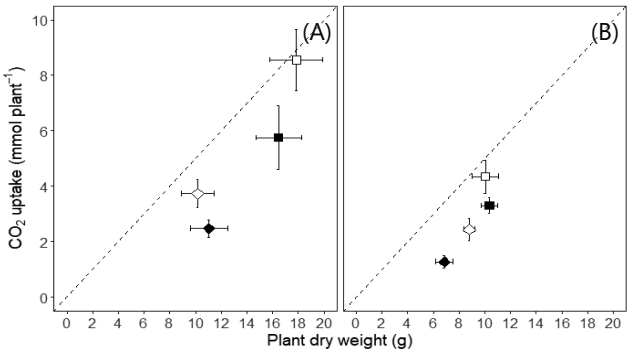

Figure 4.3 Diel cumulative CO_2 uptake per plant, as a function of plant dry weight of vegetative *Phalaenopsis* plants of two genotypes, UPO4 (A) and UPO6 (B) grown at different treatment combinations of temperature (27°C (□) or 31°C (◇)) and light (PPFD of 60 µmol m^{-2} s^{-1} (black) or 140 µmol m^{-2} s^{-1}(white)). Data represent averages (n=3) ±SEM error bars.

night, but for PC2 those were CO_2 uptake in phase I and number of leaves. For UPO6 Δ_{starch} during the day and root dry weight explained most of the variation according to PC1, but $\Delta_{sucrose}$ during the night and SLA correlated most with PC2.

The same matrix that was analysed for PCA was used for hierarchical clustering, yielding four groups with common sources of variation per genotype (Figure 4.4). This clustering is indicative for how treatments affect the different genotypes. For both genotypes, temperature seemed to be the dominating determinant of variation observed. The second order group was defined by light intensity, which differed more at 27°C than at 31°C. It is therefore not surprising that part of the variation in PCA could be linked to the treatments that were applied. PC1 largely correlated with temperature, whereas PC2 could help to explain variation caused by light intensity.

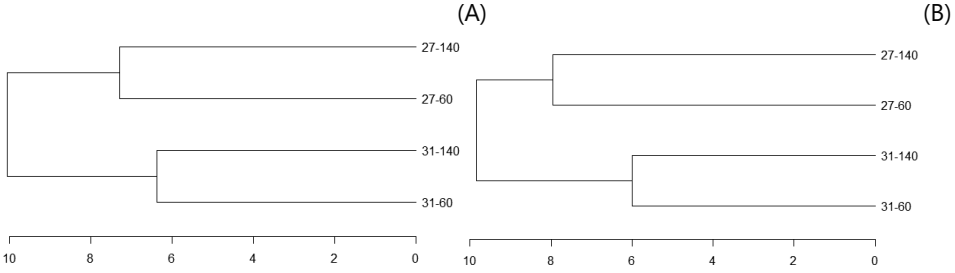

Figure 4.4 Hierarchical clustering on parameter x treatment matrix of standardized means, based on squared Euclidian distances. This was done for two *Phalaenopsis* genotypes UPO4 (A) and UPO6 (B), of which plants were grown at two temperatures (27°C or 31°C) and two light intensities (PPFD of 60 or 140 µmol m^{-2} s^{-1}).

Table 4.1 Factor loadings of principal component analysis (PCA) of *Phalaenopsis* genotypes UPO4 and UPO6, grown at different treatment combinations of temperature (27°C or 31°C) and light (PPFD of 60 or 140 µmol m^{-2} s^{-1})

	UPO4		**UPO6**	
Parameter	PC1	PC2	PC1	PC2
# Leaves	0.08193	**0.2848***	0.149	0.18426
# Old leaves	-0.183	0.16953	0.04162	0.17638
# New leaves	0.20267	0.12013	0.24098	-0.0573
Leaf area	-0.219	0.13112	-0.196	0.19859
Root DW	-0.2211	-0.1383	**-0.255**	-0.0156
Stem DW	-0.0554	0.23164	-0.1586	0.16157
Leaf DW	-0.233	0.08236	-0.235	0.11682
Shoot:root ratio	0.18992	0.18053	0.23454	0.09397
SLA	-0.156	0.22946	0.05682	**0.3024**
CO_2 uptake phase IV**	0.01795	**-0.272**	0.14285	**-0.257**
CO_2 uptake phase I	-0.0494	**-0.298**	-0.1661	-0.2316
CO_2 uptake phase II	-0.1138	**-0.254**	-0.2214	-0.155
CO_2 uptake phase III	-0.1937	-0.1761	**-0.246**	0.07472
Transpiration phase IV	0.24021	0.02216	0.24	-0.0923
Transpiration phase I	**0.2429**	-0.0176	0.16869	-0.1037
Transpiration phase II	0.0918	-0.2511	-0.0947	**-0.264**
Transpiration phase III	**0.2438**	0.0544	0.15607	-0.0157
Duration phase IV	0.1673	-0.2181	0.16533	-0.2225
Duration phase II	-0.1324	-0.2373	**-0.252**	-0.0098
Duration phase III	-0.1162	**0.2661**	-0.2155	0.14101
$\Delta_{glucose}$ (day)	**-0.241**	-0.0702	-0.0082	**0.2796**
$\Delta_{glucose}$ (night)	0.04066	0.24548	-0.1589	-0.2428
$\Delta_{fructose}$ (day)	-0.2347	-0.0991	-0.095	0.20113
$\Delta_{fructose}$ (night)	-0.0082	0.24636	-0.1519	-0.2476
$\Delta_{sucrose}$ (day)	**-0.241**	-0.04	0.1544	0.06554
$\Delta_{sucrose}$ (night)	**0.2433**	0.01997	-0.006	**-0.307**
Δ_{malate} (day)	-0.1993	-0.039	-0.1336	0.24229
Δ_{malate} (night)	0.20696	-0.1603	-0.1904	-0.2052
Δ_{starch} (day)	0.21288	-0.0868	**-0.256**	-0.0008
Δ_{starch} (night)	-0.2172	0.14687	**0.2463**	0.07396
Standard deviation	4.02742	3.28481	3.91102	3.22395
Proportion of Variance	0.54067	0.35967	0.50987	0.34646
Cumulative Proportion	0.54067	0.90034	0.50987	0.85633

* For every principal component (PC), factors of parameters with strongest loadings have been marked in bold.
** CAM-phases defined according to Osmond (1978).
Abbreviations: DW, dry weight; SLA, specific leaf area.

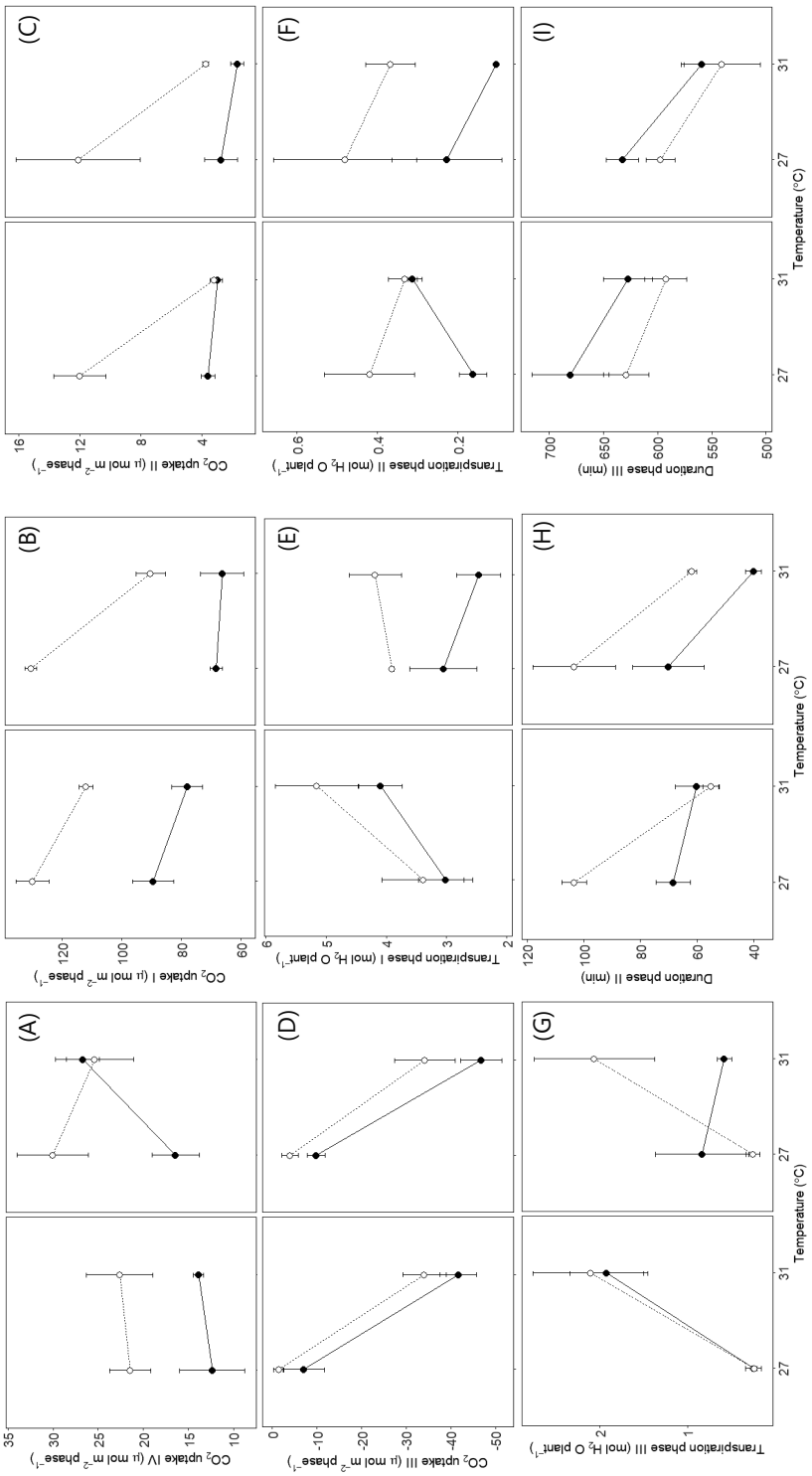

Figure 4.5 Interaction plots of photosynthesis-related parameters with strongest loadings on PC1 and PC2. (average ± SEM) of cumulative CO_2 uptake in phase IV (A), phase I (B), phase II (C), and phase III (D), transpiration in phase I (E), phase II (F) and phase III (G), as well as duration of phase II (H) and III (I) of vegetative *Phalaenopsis* plants of two genotypes, displayed per parameter UPO4 (left) and UPO6 (right), grown at different treatment combinations of temperature (27°C or 31°C) and light (PPFD of 60 µmol m^{-2} s^{-1} (closed circles) or 140 µmol m^{-2} s^{-1}(open circles).

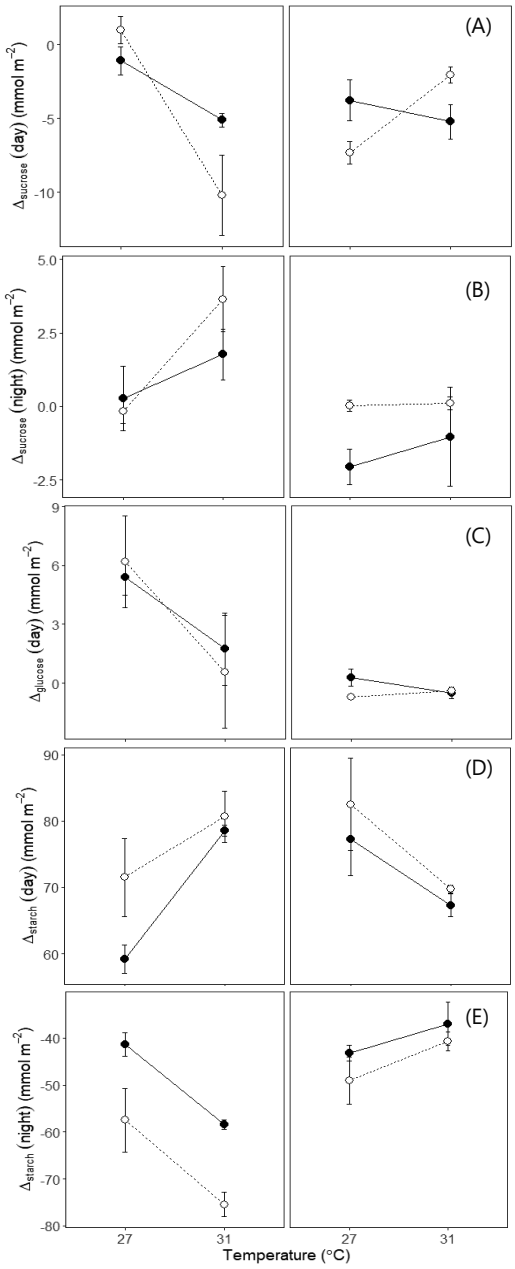

Figure 4.6 Interaction plots of carbohydrate- parameters with strongest loadings on PC1 and PC2. (average ± SEM) of $\Delta_{sucrose}$ during the day (A), and during the night (B), $\Delta_{glucose}$ during the day (C) and Δ_{starch} during the day (D) and night (E) of vegetative *Phalaenopsis* plants of two genotypes, displayed per parameter UPO4 (left) and UPO6 (right), grown at different treatment combinations of temperature (27°C or 31°C) and light (PPFD of 60 µmol m^{-2} s^{-1} (closed circles) or 140 µmol m^{-2} s^{-1} (open circles).

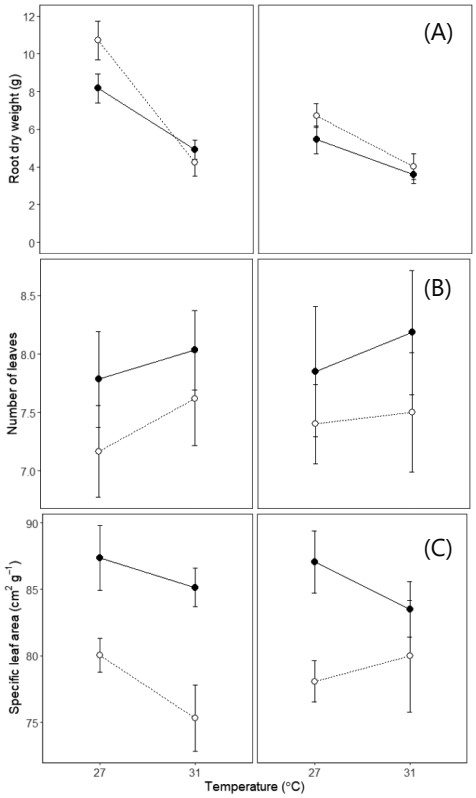

Figure 4.7 Interaction plots of biomass-related parameters with strongest loadings on PC1 and PC2. (average ± SEM) of root dry weight (A), number of leaves per plant (B), and specific leaf area (C) of vegetative *Phalaenopsis* plants of two genotypes, UPO4 (left) and UPO6 (right), grown at different treatment combinations of temperature (27°C or 31°C) and light (PPFD of 60 µmol m^{-2} s^{-1} (closed circles) or 140 µmol m^{-2} s^{-1}(open circles).

Interaction plots were created for the parameters that correlated most with each PC, for each genotype (Figure 4.5 and Figure 4.6). All parameters were significantly different between treatment and/or between genotypes. Only transpiration during phase I (during the night) was not significantly affected by light (p=0.054) nor by temperature (p=0.065). Significant three-way interactions between treatments and genotype occurred on PC1 for $\Delta_{sucrose}$ (day), duration of phase II and root dry weight, and on PC2 for SLA and transpiration (Supplemental information S4.2). Difference between genotypes became particularly clear when focussing on analysis of carbohydrates, which were contrasting in some cases. (Figure 4.6). For example, an increase in temperature resulted in an increased accumulation of starch during the day in UPO4, but a decreased accumulation of starch in UPO6 (Figure 4.7).

Interestingly, the results of metabolite analysis could not be linked directly to the results

of gas exchange. In UPO6, CO_2 uptake decreased at high temperature, in particular at high light (Figure 4.1), which was accompanied by a lower Δ_{starch} during the day (Figure 4.6). This correlated with a smaller Δ_{starch} during the night; if less starch was stored during the day, less starch was broken down during the night. However, for UPO4, a different pattern was seen: during the night, Δ_{starch} was larger at high temperature, irrespective of light intensity (Figure 4.6). This was not in line with CO_2 uptake, which was lower at high temperature (Figure 4.1).

4. Discussion

Photosynthesis is considered the primary determinant of plant growth. However, only studies on meta-level found a strong relationship between the rate of photosynthesis per unit leaf area, and plant biomass, or yield, but this was hardly the case on a species level (Poorter and Navas, 2003). Specifically for CAM plants, the relationship between rate of photosynthesis and biomass is expected to be more indirect, due to the temporal separation of CO_2 uptake and CO_2 fixation. Here, we aimed to study the physiology of CAM-species *Phalaenopsis* in response to temperature and light. We have shown that for vegetative *Phalaenopsis plants*, there is a correlation between short-term diel processes on leaf level (i.e. diel cumulative CO_2 uptake) and long-term biomass accumulation of a whole plant (Figure 4.3 and Figure 4.8). Closer inspection of the most explaining parameters revealed that the two genotypes that were used deal differently with the given combinations of temperature and light.

Recently, Hartzell et al. (2021) developed a model that combined the diel cycle of CAM plants with growth. Parameterised for CAM crops *Agave* and *Opuntia*, it makes use of an environmental productivity index based on temperature, light and precipitation (Hartzell et al., 2021). However, this model is empirical and does not determine growth based on underlying physiological processes. To overcome this limitation, we developed a conceptual framework that provides direction on how to combine data obtained at different biological and temporal scales (Chapter 2). This framework is used here to explore the different strategies genotypes use to deal with different environmental conditions (Figure 4.8). PCA showed that parameters that explained most of the variation that was found between genotypes in response to temperature and light (i.e. with the highest loading) of PC1 and PC2 generally belonged to module 1 (CAM photosynthesis) and 2 (Assimilate allocation amongst carbon pools), but not

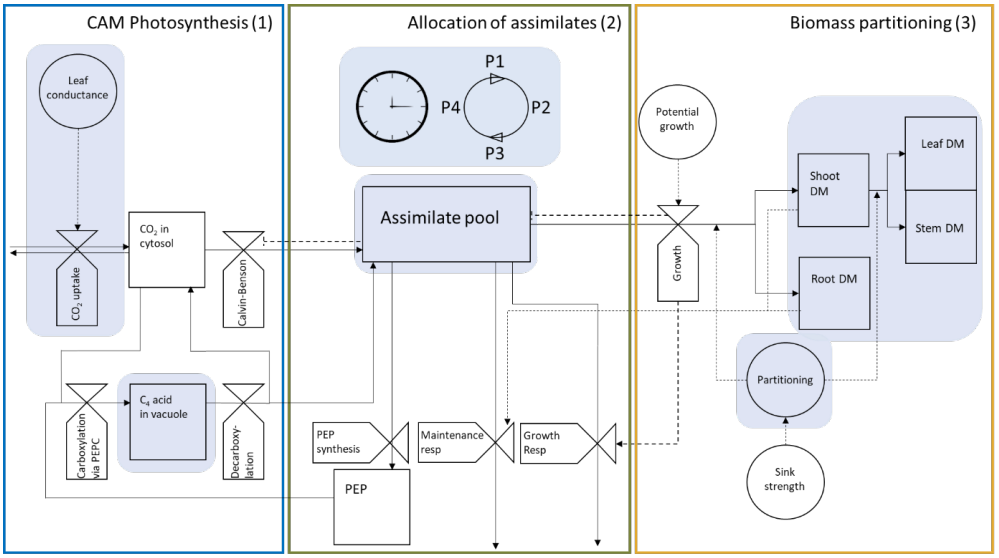

Figure 4.8 Conceptual framework for crassulacean acid metabolism (CAM) crops divided into three modules: CAM Photosynthesis (1), Allocation of assimilates (2) and Biomass partitioning (3) (adapted from Figure 2.2 in chapter 2). Forrester's (1961) symbols are used: boxes for state variables, valves for rate variables and circles for intermediate variables. Full-line arrows for carbon flows, and dashed-line arrows for information flows. Measurements were conducted in sections highlighted in blue. Abbreviations: PEP, phospho*enol*pyruvate; DM, dry matter

to module 3 (Biomass partitioning among plant organs)(Table 4.1 and Figure 4.8). Additionally, hierarchical clustering revealed that observed variation could be explained by temperature treatments first (first clustering and PC1), and then by differences in light treatments (second clustering and PC2; Figure 4.4 and Supplemental information S4.3).

4.1. CAM photosynthesis in response to temperature and light

CAM plants have evolved mainly around water-saving mechanisms. However, in a greenhouse setting this is of lesser importance, as the environment is practically non-limiting. It is therefore not strange that during breeding, genotypes are not selected based on traits that are related to survival, such as increased WUE. The results of this become apparent when studying parameters related to CAM photosynthesis in response to different environmental conditions. The contribution of different phases to cumulative CO_2 uptake varied between treatments (Figure 4.2). PCA showed that variation in CO_2 uptake of most phases of UPO4 was explained by PC2, whereas transpiration was explained better by PC1 (Table 4.1). For UPO6 however, results were not as clear-cut. In UPO6 in phase I, CO_2 uptake decreased at 31°C and 140 µmol

m^{-2} s^{-1}, whereas transpiration did not change. In plants of UPO4 at 31°C, CO_2 uptake also decreased during phase I, while transpiration increased (Figure 4.5). Interestingly, the length of a phase did not always reflect the amount of CO_2 that is taken up, nor was it in line with the amount of transpiration that occurred during that phase (Figure 4.5). In both genotypes, this will likely result in reduced WUE during phase I. Additionally, it indicates that biochemical processes, rather than stomatal opening, were limiting CO_2 uptake in phase I.

Respiratory CO_2 can function as a source of CO_2 during nocturnal CO_2 fixation in phase I, which makes it particularly difficult to determine the contribution of respiration to CO_2 fixation in CAM plants. When CAM plants were kept in CO_2-free air during the night, fixation of respiratory CO_2 was highest between 25-35°C (Winter et al. 1986). Exposure of plants to higher temperatures resulted in increased rates of respiration, although respiration rates slightly decrease again when plants acclimate to higher temperatures (Yamori et al., 2014). Thermal acclimation of respiration to higher growth temperature might not occur in UPO4, whereas it did in UPO6, because CO_2 uptake in phase I did not decrease at 140 µmol m^{-2} s^{-1} at 31°C in UPO4, but it did in UPO6 (Figure 4.5B). The lack of thermal acclimation could potentially also explain the difference in transpiration between genotypes in phase III (Figure 4.5G). In phase III when stomata are closed and decarboxylation and refixation of CO_2 occurred, transpiration was higher at 31°C, particularly in UPO4.

A large amount of CO_2 leaked out of the leaves during phase III of plants grown at 31°C (Figure 4.3 and Figure 4.5). Leakage of CO_2 occurred in both genotypes, and this leak was larger at low light. Winter et al., (1986) showed that considerable amounts of stored CO_2 can be lost in CAM plants, particularly at temperatures above 35°C. Some studies on *Phalaenopsis* showed a limited leakage of CO_2 during phase III (Lootens and Heursel, 1998, Trouwborst et al., 2014), while others found indications that at high temperatures (above 28°C) a large part of the previously stored CO_2 is lost (Arditti, 1992). In the current study, at 31°C, the leakage of CO_2 was much higher than previously reported, up to 50% of what was taken up during the other phases (Figure 4.3). In several *Kalanchoe* species, the decarboxylation rate during phase III was found to be dependent on light, which resulted in a shorter phase III and an earlier start of phase IV (Kluge, 1968, Lüttge, 2004). The reduced duration of phase III at the higher temperature treatment indicates that stored malate is depleted earlier at 31°C compared to 27°C (Figure 4.6). The (passive) efflux of malate from the vacuole is enhanced by high

temperatures (Borland and Griffiths, 1997, Lüttge, 2004). It seems that in the current study, desynchronization of the rate of malate efflux and the rate of CO_2 carboxylation in the Calvin-Benson cycle occurs, causing CO_2 to leak out of the cells.

Another possible explanation for the high amount of CO_2 leakage might be found in growing these plants at an identical day and night temperature. Yamori (2014) showed that nocturnal temperature for CO_2 fixation in CAM plants is optimal between 10-20°C and is dependent on temperature at which the plants were grown, and plant species. Others have also stated that a day-night difference in temperature of at least 10°C is needed for CAM to function properly (i.e. for nocturnal CO_2 fixation to occur) (Buchanan-Bollig and Kluge 1981, Nimmo 2000). However, differences between day and night temperatures are often very small in the humid tropics, or in the understory of a tropical forest, habitats which many CAM species often occupy (Lüttge 2004). Indeed, a recent study on *Phalaenopsis* and *K. blossfeldiana* indicates that this day-night difference is not required for all CAM species for nocturnal CO_2 uptake to take place (van Tongerlo et al., 2020)

Even though the *Phalaenopsis* genotypes that are used in this study appear to vary in the way they regulate CO_2 uptake and transpiration, it seems that they have limited ability to acclimate to a higher growth temperature, something that was also found for *K. daigremontiana* and *K. pinnata* (Yamori et al., 2014). While this limited acclimation capability would be surprising considering that these temperatures are not uncommon in the natural habitat of *Phalaenopsis*, it could be a typical result of unintentional selection in breeding.

4.2. Genotypic differences in allocation of carbohydrates

The plasticity of CAM plants to quickly respond to changes in light intensity during the day, allows CAM plants to carefully balance the amount of carbohydrates allocated towards substrate for nocturnal fixation and towards export for growth (Dodd et al., 2002). The effect of temperature and light on carbohydrate content in *Phalaenopsis* is usually studied in relation to flowering (Chen et al., 1994, Kataoka et al., 2004, Hückstädt and Torre, 2013, Lee et al., 2019). Genotypic variation in *Phalaenopsis* is apparent, not only in plant growth (Chapter 3), but also in carbohydrate content (Hückstädt and Torre, 2013). Here, we showed that responses in carbohydrate content to different climate conditions varied between genotypes. Interestingly, parameters linked to the assimilate pool that came out as highly variable for UPO4 according

to PC1, had the highest loading values found for UPO6 based on PC2 (e.g. $\Delta_{glucose}$ (day),Table 4.1), meaning that variation of those traits could be better explained by temperature in UPO4 and by light in UPO6.

It is important to note that genotypes varied not only in their response regarding carbohydrate content, but that these responses can actually be in the opposite direction . The response in starch build-up and breakdown was opposite between the genotypes that were used, in particular in response to temperature (Figure 4.6D, E). This is also apparent in the contrasting loadings of parameters according to PC1 (Table 4.1 and Supplemental Figure S4.3). The accumulation of starch during the day increased with temperature in UPO4, but decreased in UPO6. This is further reflected in starch breakdown during the night, where more starch breakdown occurred at higher temperatures in UPO4, but not in UPO6 (Figure 4.6E). The comparison of two genotypes at different biological scales makes these results particularly interesting, and raises questions on the relationships between CO_2 uptake, the assimilate pool and biomass partitioning (Figure 4.8). In UPO4, CO_2 uptake during phase I is higher at high light compared to low light, which corresponds with an increased Δ_{starch} (night) (Figure 4.5 and Figure 4.6). However, when comparing CO_2 uptake between temperature treatments, the increased Δ_{starch} (night) cannot be the result of increased CO_2 uptake during phase I, which was lower at 31°C Δ_{starch} (night) than at 27°C. As previously mentioned, it could be that respiration contributed to the large difference between Δ_{starch} (night) and CO_2 uptake during phase I. Also in UPO6, CO_2 uptake during phase I at different treatments does not correspond with the expected Δ_{starch} (night) (Figure 4.5 and Figure 4.6). Additionally, the increased turnover of starch at 31°C compared to 27°C does not result in the expected corresponding increase in biomass accumulation (Figure 4.1A,B). These results seem to invalidate arguments related to substantial carbon export out of the leaf; at least when placed in the context of export for growth. *Phalaenopsis* is a starch-storing CAM plant (note the magnitude of Δ_{starch} compared to soluble sugars in Figure 4.7), but starch alone did not account for the complete assimilate pool. In theory, all CAM plants have the potential to switch mechanisms of carbohydrate storage, as they could store their carbohydrates as starch and/or sugars (Haider et al., 2012). In practice, plants store carbohydrates either as starch, or as sugars (Christopher and Holtum, 1996).

In the current study, soluble sugars could not account for the discrepancy in CO_2 uptake, carbohydrate content, and biomass accumulation in different treatments. These

measurements were conducted on leaf samples only, but there might be a hidden role for roots, as will be discussed next.

4.3. Variation in growth and development in response to temperature and light

Previous studies on the effect of light on vegetative *Phalaenopsis* growth and development showed that, both leaf initiation and plant biomass increased with an increase in light, up to a saturating intensity , whereas SLA decreased (Figure 3.2)(Konow and Wang, 2001, Lee et al., 2019). Here, leaf related traits, such as number of leaves (UPO4) and SLA (UPO6) correlated with PC2 (Table 4.1). The former was surprising, because the absolute difference between the lowest and highest number of leaves was merely 0.6 leaves (Figure 4.7B), whilst leaf number still had a high loading value for PC2 and was significantly different between light and temperature treatments (Table S4.1). These results are in line with previous studies on *Phalaenopsis* (Konow and Wang, 2001, Lee et al., 2019). SLA varied between genotypes in response to both temperature and light (Figure 4.7C and Table S4.1). SLA affects all the previously discussed parameters related to gas exchange and biochemistry, which are expressed on a per-area basis. Thicker leaves are more likely to have a higher capacity for storage of carbohydrates. Therefore, while expressed on a per-area basis, we corrected for differences in leaf thickness and subsequent differences in storage capacity based on weight of the samples.

Root dry weight for UPO6 decreased with an increase in temperature (Table 4.1 and Figure 4.7). Also root dry weight of UPO4 decreased strongly with an increase in temperature. Shoot:root ratio tends to decrease with light and increase with temperature in *Phalaenopsis* (Chapter 3). This was also the case for both genotypes studied here (Figure 4.7). Most studies on *Phalaenopsis* show an increase in total plant biomass due to light intensity (Chapter 3, Trouwborst et al. 2016*b*) in different growth phases. Increased root growth could enhance storage capacity of carbohydrates. Lee and Wang (1997) showed that, compared to leaves, roots of vegetative *Phalaenopsis* plants contained two- or trifold the level of soluble sugars. Carbohydrate content in roots was at least 70% higher than in leaves. Upon flowering, root- (and leaf) carbohydrate content decreased quickly, suggesting their storage function in the vegetative phase. Larger roots could mean a larger storage capacity for carbohydrates, e.g. in

Figure 4.9 Honeydew secretion of a *Phalaenopsis* leaf.

the case of UPO4 at 27°C compared to UPO4 at 31°C. Additionally, accumulation of carbohydrates in leaves of *Phalaenopsis* induced some honeydew secretion (Figure 4.9)(Xu et al., 2017), which might have caused a loss of carbohydrates and thus affected the carbon budget significantly.

Measurements of carbohydrates can be used to calculate carbon budgets (Ceusters et al., 2010, Haider et al., 2012), which can give insight in the mechanism of CAM from CO_2 uptake to growth. Here, we are not be able to finalize the carbon budgets for different genotypes that were grown at various environmental conditions. Apart from the limited number of carbohydrates measured, commonly made assumptions for carbon budgets do not necessarily seem to apply to *Phalaenopsis*. For instance, which pools should be accounted for, e.g. for export for respiration and biomass, are ambiguous as the destination can be multiple and change per phase. This calls for caution when creating a carbon budget to explain carbohydrate partitioning in *Phalaenopsis*. Given the potential role of roots in carbohydrate storage, further research on *Phalaenopsis* physiology could benefit from a stronger focus on roots.

5. Conclusion

Here, we aimed to explain differences in growth and development in *Phalaenopsis* plants of two genotypes in response to a combination of four temperature and light treatments. Plant dry weight decreased with an increase in temperature in both genotypes. While some interaction in the effect of light and temperature on plant dry weight occurred in UPO4, differences were small (Figure 4.1). At 27°C, UPO4 seemed to perform better than UPO6 with

regards to biomass accumulation, but at 31°C there were no differences between genotypes. While diel cumulative CO_2 uptake per plant correlated with plant dry weight (Figure 4.3), analysis of other measured and derived parameters did not result in an unequivocal, predictive link between CAM photosynthesis measurements, assimilate allocation among carbon pools, and allocation of biomass towards different plant organs or plant growth (Figure 4.8). The relation between parameters varied with genotype and treatment combination, which means that the genotypes varied in how they responded to the environment and how they achieved their final biomass. Results indicate that there might be an important, but so far hidden role of roots in the carbon budget of *Phalaenopsis*, which requires further study. Using a framework to structure the analysis of short-term and long-term measurements as is done here, can help to increase insight in how physiological processes contribute to plant growth. This is particularly useful for ideotype breeding, where selecting relevant individual traits from different genotypes based on ecophysiology can help to develop improved cultivars (Martre et al., 2015). This work could help breeders to prevent that they – inadvertently – select for undesirable physiological traits (such as reduced WUE) in their quest for the perfectly flowering *Phalaenopsis*.

6. Acknowledgements

We are grateful for the help of Arjen van de Peppel on biochemical analysis, and Joke Oosterkamp for helping with setting up the experiment and with collecting data. A special thanks goes to Jan Sommeijer who was of tremendous help with experimental work and collecting data, as part of his MSc thesis at Wageningen University.

Supplemental information S4

S4.1 Biplot from PCA

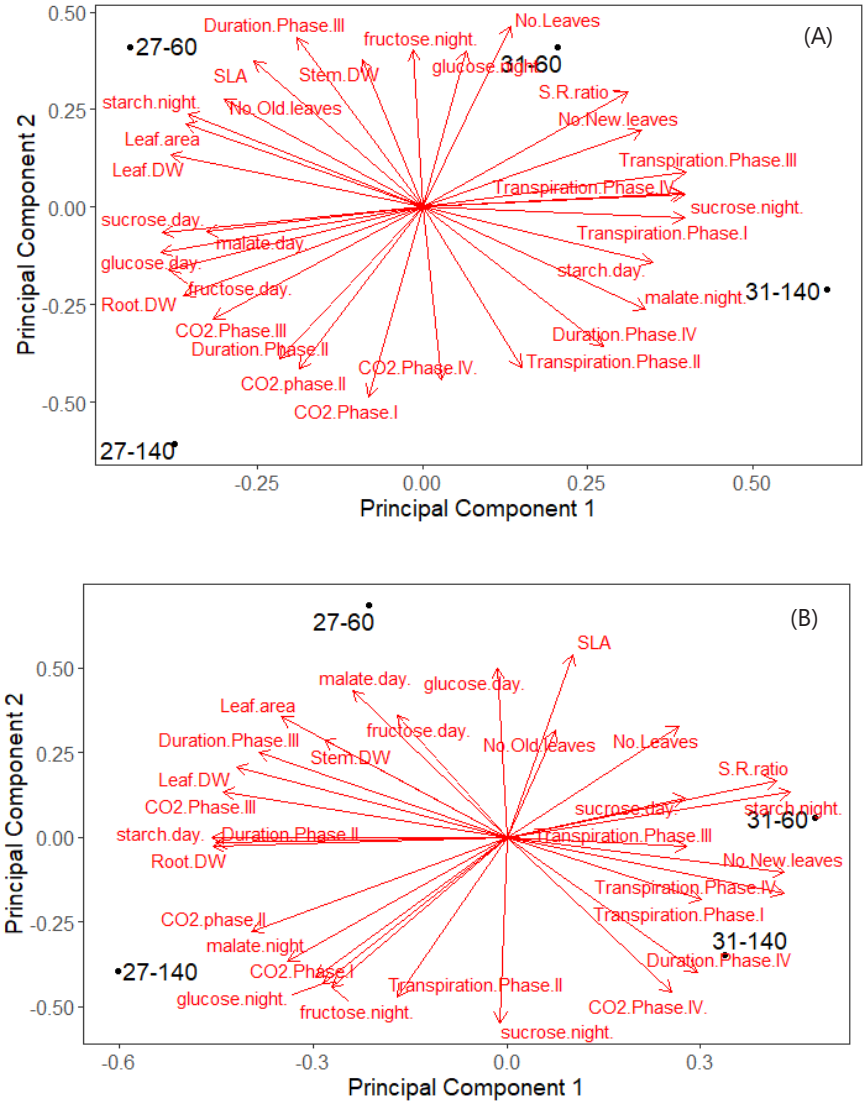

Figure S4.3 Biplots showing the variation of each parameter in relation to environmental conditions, of two vegetative *Phalaenopsis* genotypes, UPO4(A) and UPO6 (B). Plants were grown for 28 weeks at different combinations of temperature (27°C (LT) or 31°C (HT)) and light (PPFD of 60 µmol m^{-2} s^{-1} (LL) or 140 µmol m^{-2} s^{-1} (HL)).

S4.2 P-values per parameter

Table S4.1 P-values of three-way ANOVA of plant parameters that were used in principal component analysis (PCA) of two cultivars, grown at two temperatures x two light intensities

Parameter	Temp	Light	UPO	Temp × Light	Temp × UPO	Light × UPO	Temp × Light × UPO
# Leaves	0.045	0.000	0.552	0.952	0.634	0.858	0.441
# Old leaves	0.105	0.000	0.097	0.501	0.157	0.271	0.025
# New leaves	0.000	0.444	0.576	0.962	0.121	0.472	0.291
Leaf area	0.000	0.697	0.000	0.105	0.000	0.183	0.005
Root DW	0.000	0.304	0.000	0.196	0.000	0.086	0.013
Stem DW	0.558	0.141	0.000	0.349	0.055	0.178	0.738
Leaf DW	0.005	0.121	0.000	0.308	0.072	0.000	0.033
Shoot:root ratio	0.000	0.000	0.000	0.656	0.000	0.240	0.574
SLA	0.082	0.000	0.776	0.509	0.065	0.105	0.007
CO_2 phase IV*	0.851	0.020	0.054	0.414	0.729	0.932	0.425
CO_2 phase I	0.008	0.000	0.005	0.047	0.355	0.382	0.081
CO_2 phase II	0.006	0.003	0.059	0.066	0.679	0.039	0.215
CO_2 phase III	0.000	0.041	0.236	0.318	0.687	0.425	0.438
Transpiration phase IV	0.012	0.051	0.314	0.092	0.183	0.322	0.529
Transpiration phase I	0.162	0.054	0.202	0.352	0.065	0.459	0.903
Transpiration phase II	0.947	0.022	0.330	0.510	0.233	0.191	0.225
Transpiration phase III	0.007	0.517	0.581	0.120	0.093	0.632	0.108
Duration phase IV	0.022	0.008	0.004	0.813	0.969	0.235	0.547
Duration phase II	0.007	0.029	0.162	0.269	0.206	0.033	0.024
Duration phase III	0.044	0.139	0.009	0.684	0.508	0.591	1.000
$\Delta_{glucose}$ (day)	0.149	0.748	0.002	0.675	0.160	0.452	0.913
$\Delta_{glucose}$ (night)	0.806	0.711	0.034	0.376	0.178	0.418	0.361
$\Delta_{fructose}$ (day)	0.142	0.675	0.019	0.957	0.130	0.806	0.717
$\Delta_{fructose}$ (night)	0.738	0.239	0.289	0.961	0.671	0.761	0.445
$\Delta_{sucrose}$ (day)	0.114	0.909	0.443	0.836	0.009	0.319	0.038
$\Delta_{sucrose}$ (night)	0.243	0.735	0.007	0.801	0.074	0.654	0.208
Δ_{malate} (day)	0.060	0.926	0.076	0.113	0.161	0.847	0.674
Δ_{malate} (night)	0.625	0.150	0.125	0.691	0.012	0.535	0.312
Δ_{starch} (day)	0.717	0.228	0.658	0.441	0.014	0.666	0.631
Δ_{starch} (night)	0.369	0.107	0.018	0.927	0.035	0.231	0.930

*CAM-phases defined according to Osmond (1978).
Abbreviations: DW, dry weight; SLA, specific leaf area.

Chapter 5

Crassulacean acid metabolism species differ in the contribution of C_3 and C_4 carboxylation to end of day CO_2 fixation

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Abstract

Crassulacean acid metabolism (CAM) is a photosynthetic pathway that temporally separates the nocturnal CO_2 uptake, via phosphoenolpyruvate carboxylase (PEPC, C_4 carboxylation), from the diurnal refixation by Rubisco (C_3 carboxylation). At the end of the day (CAM-Phase IV), when nocturnally stored CO_2 has depleted, stomata reopen and allow additional CO_2 uptake, which can be fixed by Rubisco or by PEPC. This work examined the CO_2 uptake via C_3 and C_4 carboxylation in phase IV in the CAM species *Phalaenopsis* 'Sacramento' and *Kalanchoe blossfeldiana* 'Saja'. Short blackout periods during phase IV caused a sharp drop in CO_2 uptake in *K. blossfeldiana* but not in *Phalaenopsis*, indicating strong Rubisco activity only in *K. blossfeldiana*. Chlorophyll fluorescence revealed a progressive decrease in ΦPSII in *Phalaenopsis*, implying decreasing Rubisco activity, while ΦPSII remained constant in phase IV in *K. blossfeldiana*. However, short switching to 2% O_2 indicated the presence of photorespiration and thus Rubisco activity in both species throughout phase IV. Lastly, in *Phalaenopsis,* accumulation of starch in phase IV occurred. These results indicate that in *Phalaenopsis*, PEPC was the main carboxylase in phase IV, although Rubisco remained active throughout the whole phase. This will lead to double carboxylation (futile cycling) but may help to avoid photoinhibition.

Keywords: Crassulacean acid metabolism (CAM), *Kalanchoe, Phalaenopsis,* phospho*enol*pyruvate carboxylase (PEPC), Rubisco

1. Introduction

Crassulacean acid metabolism (CAM) is a specialized photosynthetic pathway with an inverse day/night pattern of stomatal opening. CAM plants temporally separate CO_2 fixation during the night from refixation via the Calvin-Benson cycle behind closed stomata during the daytime. Osmond (1978) introduced a framework to distinguish the different processes taking place over a diel cycle in a CAM plant. The nocturnal period in which stomata are open is referred to as phase I. During this phase, phosphoenolpyruvate carboxylase (PEPC) catalyses atmospheric or respiratory CO_2 (as HCO_3^-) to bind with phosphoenolpyruvate (PEP), which is stored in the vacuole as a C_4 acid (Lüttge, 2001). The refixation period during the day, when stomata are closed, is referred to as phase III. In between these two phases with distinct carboxylation processes, transitional phases (II and IV) may occur under environmentally favourable conditions (Dodd et al., 2002). During phase II at dawn, the transition from CO_2 carboxylation via PEPC to carboxylation via Rubisco occurs. PEPC carboxylation may continue at dawn while Rubisco is activated to avoid photoinhibition (Roberts et al., 1997). Phase II is defined to last until stomata close. At the end of phase III, C_i decreases due to malate depletion, causing stomata to reopen. This marks the start of phase IV, in which CO_2 uptake takes place again (Winter and Smith, 1996, Males and Griffiths, 2017). Phase IV lasts until dark.

Diel regulation of the two carboxylases is essential for the functioning of CAM. Carbon isotope discrimination data confirmed that PEPC and Rubisco can be simultaneously active in both phases II and IV (Ritz et al., 1986, Roberts et al., 1997). Gas exchange measurements on CO_2 uptake and stomatal conductance are most commonly used to determine diel patterns and identify CAM-phases. To determine the contribution of different carboxylation pathways on CO_2 uptake, Winter and Tenhunen (1982) used a combination of ambient and low CO_2 and O_2 concentrations while measuring CO_2 gas exchange, and found that both PEPC and Rubisco are active during phase II. Also, chlorophyll fluorescence measurements can be used to distinguish between carboxylases. Griffiths et al. (2008) showed that there is a direct positive correlation between Rubisco activity, Rubisco activation and the electron transport rate (ETR). Carboxylation via PEPC in the light can also be considered an indirect electron sink, but not a strong one (Maxwell et al., 1999). PEPC carboxylation activity is associated with low ETR and high non-photochemical quenching (NPQ) (Griffiths et al., 2008). NPQ mechanisms safely

dissipate excess excitation energy that cannot be used for photochemistry (Demmig-Adams and Adams, 2006). Low ETR and high NPQ typically occur in CAM plants at the beginning and the end of the day under natural conditions, when PEPC may be active. Gas exchange measurements at different CO_2 and O_2 concentrations combined with chlorophyll fluorescence can be used as a non-invasive and non-destructive measurement to determine which carboxylation pathway is likely to be active.

Previously it was assumed that the transitional phases II and IV do not contribute much to carbon gain (Borland and Griffiths, 1996). Later on, in particular phase IV was recognized for contributing substantially to the total carbon uptake under well-watered conditions (Dodd et al., 2002), which could help increase productivity. While CAM has the potential of high yields, it is typically not seen as a trait that is favourable for crops in agriculture (Davis et al., 2019). CAM has a higher energetic cost per fixed CO_2 due to temporally separating two CO_2 fixation steps. While this might seem significant, the higher energetic cost results in a trivial productivity penalty compared to C_3 plants (Nobel, 1991, Shameer et al., 2018). The limited penalty on productivity is considered to be mainly due to the suppression of photorespiration, which can increase the cost of CO_2 fixation in C_3 plants by 25% (Nobel, 1991). On the other hand, direct CO_2 fixation via Rubisco in CAM plants in phases II and IV may go hand in hand with significant photorespiration (Borland et al., 2000). The succulent structure of CAM leaves results in reduced intercellular space (Nelson and Sage, 2008), which reduces the internal conductance for CO_2. Maxwell et al. (1997) showed for *Kalanchoe daigremontiana* that CO_2 concentration at the carboxylation site for Rubisco can be as low as 110 ppm during phase IV. For that reason, Rubisco would be less effective for CO_2 fixation compared to PEPC, which has a lower compensation point (Ceusters and Borland, 2010). If uptake by PEPC in phase IV is supplementary to nocturnal uptake, it could bypass photorespiration and result in additional CO_2 uptake. It is generally believed that, in phase IV, CO_2 is mainly fixed via Rubisco (Osmond and Holtum, 1981, Ceusters et al., 2010, Cushman, 2017). Also, the carbon that is fixed during this phase is largely partitioned for growth (e.g. Dodd et al., 2002; Maxwell et al., 1999; Nobel, 1996), which could boost the productivity of CAM plants. More recently, Ceusters et al. (2010) already questioned these statements by showing that, for the CAM bromeliad *Aechmea,* the CO_2 uptake in phase IV can be dominated by PEPC, and that phase IV activity is not explanatory for plant growth rates and biomass accumulation.

The main aim of this study was to investigate the extent to which C_3 and C_4 carboxylation in CAM-phase IV contribute to CO_2 uptake. We studied two obligate CAM species: *Phalaenopsis* 'Sacramento' and *Kalanchoe blossfeldiana* 'Saja'. *Phalaenopsis*, in particular, is considered economically important in the horticultural sector (Davis et al., 2019). The *Kalanchoe* genus, in general, is relatively well studied due to its potential as CAM model plant and its wide range of CAM phenotypes, ontogenetically shifting from C_3 to CAM, makes it an interesting object of study (Yang et al., 2015, Winter, 2019). Species used in this study are both cultivated for their ornamental value. *Phalaenopsis* is an epiphytic orchid that can be mainly found in forests in tropical Asia (Tsai, 2011), whereas *K. blossfeldiana* originates in Madagascar, where it grows at higher altitudes and in humid forests at moderate temperatures (Smith et al., 2019). In *K. blossfeldiana*, short days induce flowering and accelerate the switch to CAM (Winter and Holtum, 2014). In the current study, these plants were grown in climate chambers. We showed that CO_2 taken up in phase IV in *K. blossfeldiana* 'Saja' was mainly fixed via Rubisco, while PEPC seemed to be the main carboxylase in *Phalaenopsis* 'Sacramento'. However, in *Phalaenopsis,* Rubisco remained active as well, which may lead to futile cycling because of double carboxylation but could help to avoid photoinhibition.

2. Material and methods

2.1. Plant material and growth conditions

Vegetative *Phalaenopsis* hybrid cv. 'Sacramento' (further referred to as *Phalaenopsis*) and *Kalanchoe blossfeldiana* cv. 'Saja' (further referred to as *K. blossfeldiana*) were grown in two separate climate chambers. Plants were grown in the greenhouse before transfer to the climate chambers. Vegetative *Phalaenopsis* plants were potted in 12 cm pots and grown in a Venlo type greenhouse (Bleiswijk, The Netherlands) for 21 weeks, after propagation in the lab. Cuttings of *K. blossfeldiana* plants were potted in 10.5 cm pots and grown for 8 weeks, of which 5 weeks in short-day conditions, in a Venlo type greenhouse ('s Gravenzande, The Netherlands). Plants were allowed to acclimatise to climate chamber conditions for one week before starting measurements. In the time range where the experimental work was conducted, *K. blossfeldiana* plants were expected to show maximum CAM functioning and malate carboxylation (40-60 days, Queiroz and Morel, 1974). *Phalaenopsis* was grown at a temperature of 27°C and PPFD

of 140 µmol photons m^{-2} s^{-1} between 05:00 and 21:00. *K. blossfeldiana* was grown at 20°C with a PPFD of 140 µmol photons m^{-2} s^{-1} between 08:00 and 18:00. Growth conditions for each species were comparable to those applied by commercial growers. The shorter day length used for growth of *K. blossfeldiana* induces CAM in young leaves, as well as flowering (Queiroz and Morel, 1974). Plants were grown under red/white LED modules with supplementary far-red (23 µmol photons m^{-2} s^{-1}) (Philips GreenPower LED production module deep red/white and GreenPower LED research module Far Red) at a photostationary state of 0.83 (Sager et al., 1988)(Supplemental information S3.1). Spacing was done in such a way that no shading due to nearby plants occurred. For both species, CO_2 concentration was kept at 600 ppm, as is common practice in commercial greenhouses, and vapor deficit of the air at 7.2 g m^{-3}. Plants were watered every five days with a nutrient solution that had an EC 1.2 mS cm^{-1} and pH of 5.7. (Supplemental information S3.2). For all measurements, either the youngest mature leaf (*Phalaenopsis)* or the third leaf pair from the apex, present at the start of the experiment (*K. blossfeldiana),* was used.

2.2. Gas exchange and chlorophyll fluorescence measurements

Gas exchange measurements were done using LI-6400XT (Li-Cor Inc., Lincoln) using the LCF chamber with a flow rate of 200 µmol m^{-2} s^{-1}, light intensity of 140 µmol photons m^{-2} s^{-1} (10% blue), block controlled temperature of either 27°C (*Phalaenopsis*) or 20°C (*K. blossfeldiana*). CO_2 was controlled at 600 ppm. To ensure continuous and steady CO_2 supply, an external gas cylinder and a setup to humidify the air to ambient conditions were used. For chlorophyll fluorescence measurements, a rectangular flash was used. Intensities of the 0.8 s saturating flash (>6200 µmol photons m^{-2} s^{-1} for *Phalaenopsis* and >6300 µmol photons m^{-2} s^{-1} for *K. blossfeldiana*) and measuring light were tested beforehand to avoid photo-inhibition and reduction of Q_A, respectively. Measurements were made in situ in the climate chamber.

2.2.1. Diel profiles

Data were collected starting in phase III and continued throughout the subsequent night and morning under conditions similar to those in the climate chamber to record the 'steady-state' data of plants (n=8). Data were logged every minute, while matching of IRGAs was done every five minutes. For readability of the figures, plotted results contain one data point every ten

minutes. Each data point represents a single measured value. During the day, chlorophyll fluorescence measurements were made every 15 minutes, in the night every hour. These data were also used to determine the beginning of phase IV. When the net CO_2 uptake rate was higher than 0.1 µmol CO_2 m^{-2} s^{-1} for at least 3 consecutive minutes and remained positive, this was marked as the start of phase IV.

2.2.2. Procedures to distinguish C_3- and C_4-driven CO_2-uptake during phase IV

Short blackout period

Light in the leaf chamber was switched off for five minutes three (*K. blossfeldiana*: 210, 120 and 30 minutes before lights off) or four (*Phalaenopsis*: 300, 210, 120 and 30 minutes before lights off) times during the progression of phase IV. Data were logged every minute, but from ten minutes before until ten minutes after lights off, the frequency of logging was increased to every five seconds. When logging data under changing conditions, the lag of the system to changes in water vapor content has to be considered. This is especially true for changes in stomatal conductance caused by disabling C_3 uptake due to switching lights off. Therefore, from the high logging frequency only one data point per 100 seconds was used in data analysis and figures. To ensure that switching off the lights was not the reason for PEPC induction, a separate set of measurements was conducted, where lights were only switched off at the last time point (Supplemental information S5.3).

2% O_2 measurements

In addition to short blackout-period measurements, the part of the leaf clamped in the chamber was subjected to 2% O_2. This was done to 1) determine whether photorespiration occurred, and (2) determine the contribution of Rubisco/PEPC carboxylation throughout phase IV. Settings in the leaf chamber were as described previously. The leaf chamber was sealed 15 minutes prior to the start of the first measurement. During every measurement, IRGAs were matched and gas exchange and fluorescence data were logged. After an initial steady-state measurement, CO_2 was mixed with 2% O_2, using a separate gas mixing system with mass flow meters. Plants were exposed to this air mixture for 5 minutes, after which data were logged again. This was done twice for *K. blossfeldiana* (150 and 45 minutes before lights off) and three

times for Phalaenopsis (255, 150 and 45 minutes before lights off) (n=8). New plants were used at every time step. ΦPSII was calculated according to Baker (2008). To calculate NPQ, a dark-adapted leaf is required, in which NPQ is fully relaxed. However, Tietz et al. (2017) demonstrated that NPQ can also be derived without having a fully dark-adapted leaf. This new fluorescence parameter is characterized as $NPQ_{(T)}$ (Eq. 5.1) and was used accordingly in the current study.

$$NPQ_{(T)} = \frac{4.88}{\left(\frac{Fl_m}{Fl_0}\right)-1} - 1 \qquad \text{Eq. 5.1}$$

2.3. Sampling and analysis of metabolites

Leaf discs were taken for biochemical analysis at several time points throughout phase IV, and immediately after lights off. For *K. blossfeldiana*, sampling in phase IV was done every hour, i.e. three, two and one hour before lights off. *Phalaenopsis* was sampled every two hours, i.e. six, four and two hours before lights off. New plants were used at every time point. At each time point, we measured 3 different plants (3 biological replicates; n=3). Per plant three leaf discs were taken (3 sampling replicates per biological replicate) and immediately placed in liquid nitrogen before placing them in the -80°C freezer. Leaf discs were freeze-dried and ground to powder with two metal pellets (3 mm) in an Eppendorf for a minute using a ball mill. Samples were stored in a desiccator until further processing.

2.3.1. Sample preparation for anion and sugar analysis

The freeze-dried powder (15 mg) was dissolved in 5 mL 75% ethanol. Samples were heated in a shaking water bath at 80°C for 20 minutes and centrifugated for 5 minutes (8500 g, 4°C, Universal 320R, Hettich). 1 mL of supernatant was dried in the Speed Vac at 55°C for two hours or until dry. The evaporated ethanol was replaced by 1 mL of MiliQ water, and mixed for 10 min in an ultrasonic bath at 50 Hz, followed by vortex and centrifuge for 10 min. This protocol was used for both analyses of anions and sugars. Samples were then diluted, 5 times for anions and 10 times for soluble sugars.

2.3.2. Sample preparation for starch analysis

Of the previously prepared samples for anions and sugars, the remaining supernatant was discarded and 3 mL of 80% ethanol was added. Samples were centrifugated 15 min (8500 g, 4°C), then washed twice with 80% ethanol. After discarding the supernatant again, pellets were placed in the Speed Vac at 55°C for 25 min. After drying, 2 mL of thermostable α-amylase solution (1 mg Rohalase mL^{-1}) was added to the pellet, and the sample was placed in a shaking water bath at 90°C for 30 min. 1 mL of amyloglucosidase (0.5 mg mL^{-1} citrate buffer, 50 mM at a pH of 4.6) was added, again placed in a shaking water bath at 60°C for 15 min. 1 mL was put into an Eppendorf tube that was centrifuged for 15 min (8500 g, 4°C) and diluted 20 times before analysis.

2.3.3. Sample analysis

Soluble sugars and starch were quantified using High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD; Dionex ICS5000, Thermo Fisher Inc.), equipped with a CarboPac1 column (250 × 2 mm) eluted with 100 mM NaOH. Anions were quantified using a Dionex HPAEC system (Dionex Corporation) equipped with a GS50 pump, an ED50 detector and with an IonPac AS11-HC (250x2mm) column and eluted with a gradient starting at 16 mM NaOH. The total accumulation of malate, citrate, sucrose, fructose, glucose and starch was calculated in mmol m^{-2} using leaf mass area of dried leaf discs.

2.4. Statistical analysis

Measurements at 2% O_2 were analysed using a paired t-test for samples within one species at one timepoint. Simple linear regression was conducted on the results of metabolite analysis at different time points in phase IV. For all analyses, Genstat 19th edition (VSN International Ltd) was used. Treatment effects within species were considered significant at $p<0.05$. All data are expressed as mean ± standard error of the mean.

3. Results

3.1. Diel profiles of CO_2 uptake

When lights were switched off (start of phase I), there was a direct, sharp drop in net CO_2 uptake in *K. blossfeldiana* 'Saja', even resulting in the release of CO_2 for the first few hours of phase I (Figure 5.1A). This indicated a strong Rubisco-mediated uptake during phase IV, and negligible PEPC-mediated CO_2-uptake. After a few hours, net CO_2 uptake became positive, but CO_2 uptake rates stayed low during the night period. In *Phalaenopsis* 'Sacramento', transitioning from light to dark did not result in a decrease in the CO_2 uptake rate (Figure 5.1B). Instead, an increase occurred during the first hour after the lights switched off. This indicates a large contribution of PEPC-mediated CO_2 uptake in phase IV, which is opposite to *K. blossfeldiana*. Fluorescence measurements showed that the photosynthetic operating efficiency (ΦPSII)(Baker, 2008) decreased progressively in *Phalaenopsis* throughout phase IV, which implies a decreasing Rubisco activity over time. However, *K. blossfeldiana* maintained a constant photosynthetic efficiency until the end of the day (Figure 5.2).

3.2. Multiple short blackout periods to distinguish C_3- and C_4-driven CO_2-uptake during phase IV

Momentarily switching off the light throughout phase IV always resulted in a reversal of CO_2-exchange direction from uptake towards release in *K. blossfeldiana*, which suggests that all CO_2 uptake in phase IV is via C_3 photosynthesis (Figure 5.3). The immediate drop in CO_2 exchange was followed by a delayed closing response of the stomata (Figure 5.3B,C). Stomatal closure was not as visible the first time, most likely because stomatal conductance was very low (Figure 5.3A). After lights on, CO_2 uptake was not immediately back at the same rate as before (when lights were off) due to a delayed response of the stomata. Stomatal conductance started to decrease a few minutes after lights were switched off but took more than 20 minutes to fully recover. In *Phalaenopsis*, CO_2 uptake dropped when lights switched off at the beginning of phase IV (Figure 5.4A,B) but not later on (Figure 5.4C,D). During the second drop, there was a decrease in CO_2 exchange, but the net uptake remained positive, suggesting PEPC-carboxylation was already induced. At the last two time points, there was no decrease in CO_2

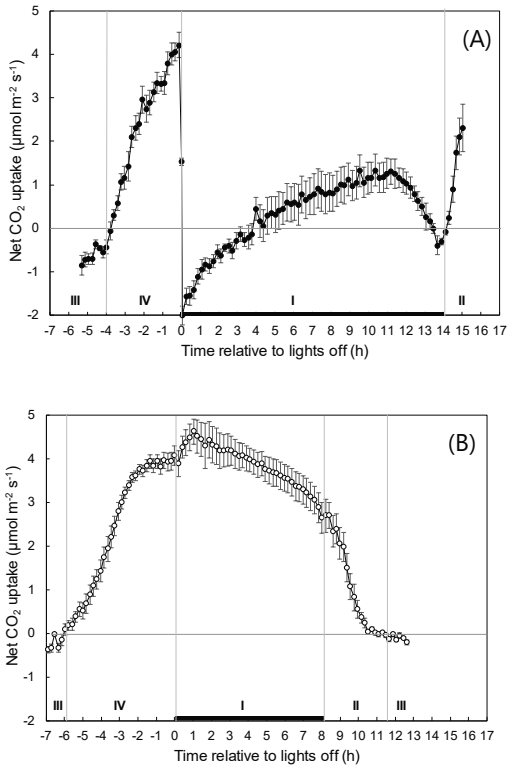

Figure 5.1 Diel profiles of CO_2 exchange rates of *Kalanchoe blossfeldiana* cv. Saja (A) and *Phalaenopsis* cv. Sacramento (B) leaves. The third leaf pair from the apex of *K. blossfeldiana* and the youngest mature leaf of *Phalaenopsis* were used. Solid bar on x -axis indicates night period. CAM phases, as described by Osmond (1978), are indicated by roman numerals. Data represent the average of 8 replicates with SEM. Plants were grown in climate chambers at 660 ppm CO_2 and a PPFD of 140 µmol photons m^{-2} s^{-1} with additional far-red of 23 µmol photons m^{-2} s^{-1}. *K. blossfeldiana* was grown at 20°C and a day length of 10 hours, and *Phalaenopsis* was grown at 27°C and a day length of 16 hours.

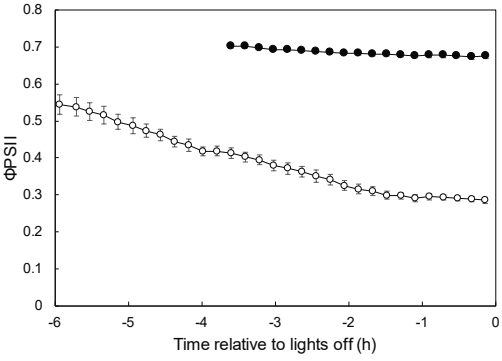

Figure 5.2 Photosynthetic operating efficiency (ΦPSII) in *K. blossfeldiana* (closed circles) and *Phalaenopsis* (open circles) leaves in phase IV. Data represent the average of 8 replicates with SEM. Plant growth conditions as described in Figure 5.1.

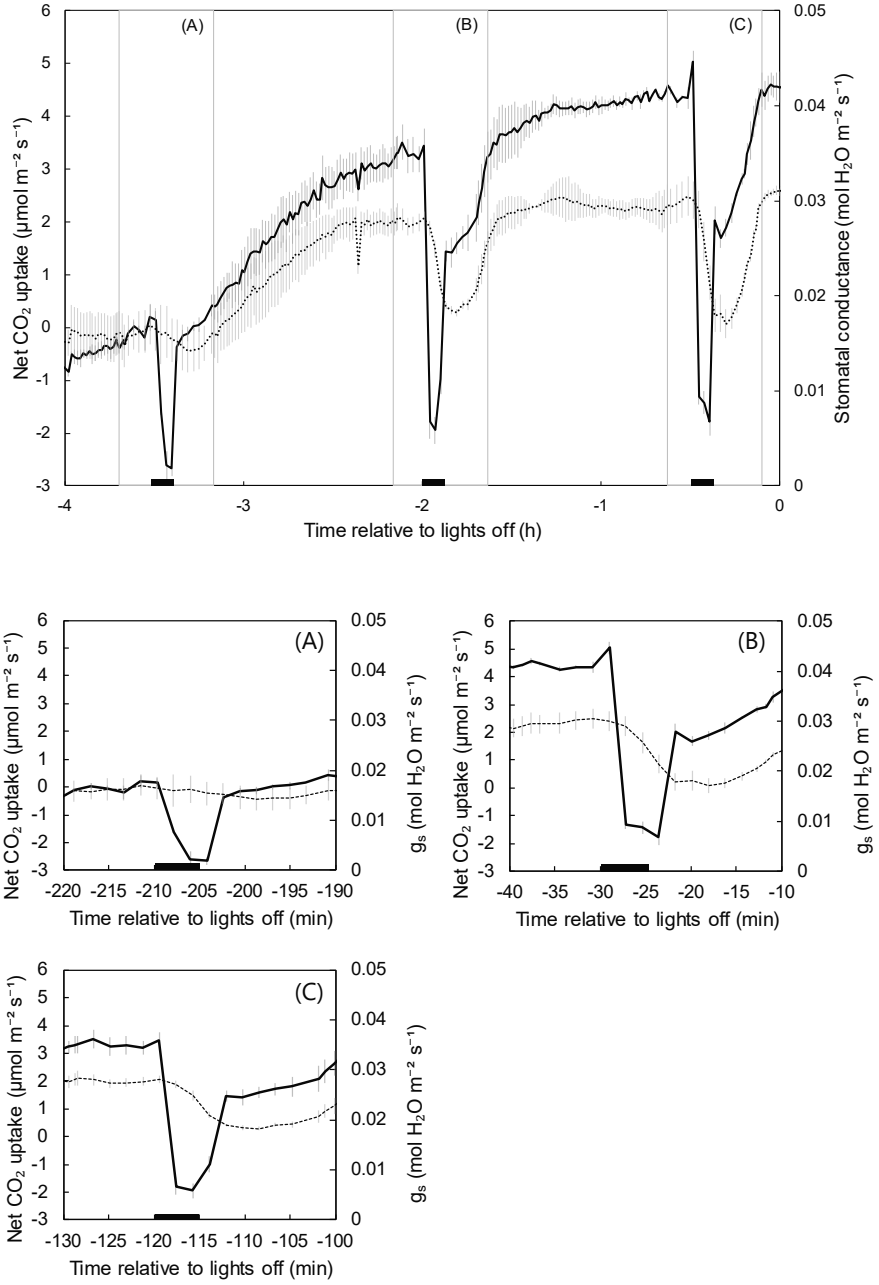

Figure 5.3 Response of CO_2 uptake (solid line) and stomatal conductance (g_s, dashed line) of *K. blossfeldiana* leaves (third leaf pair from the apex) to a 5-minute dark period in phase IV. 3.5 h (A), 2h (B) or 0.5 h (C) before end of day, the light in the leaf chamber was switched off for 5 minutes before returning to 140 µmol photons m^{-2} s^{-1}, as indicated by solid black bar. Data represent the average of 3 replicates with SEM. Plant growth conditions as described in Figure 5.1.

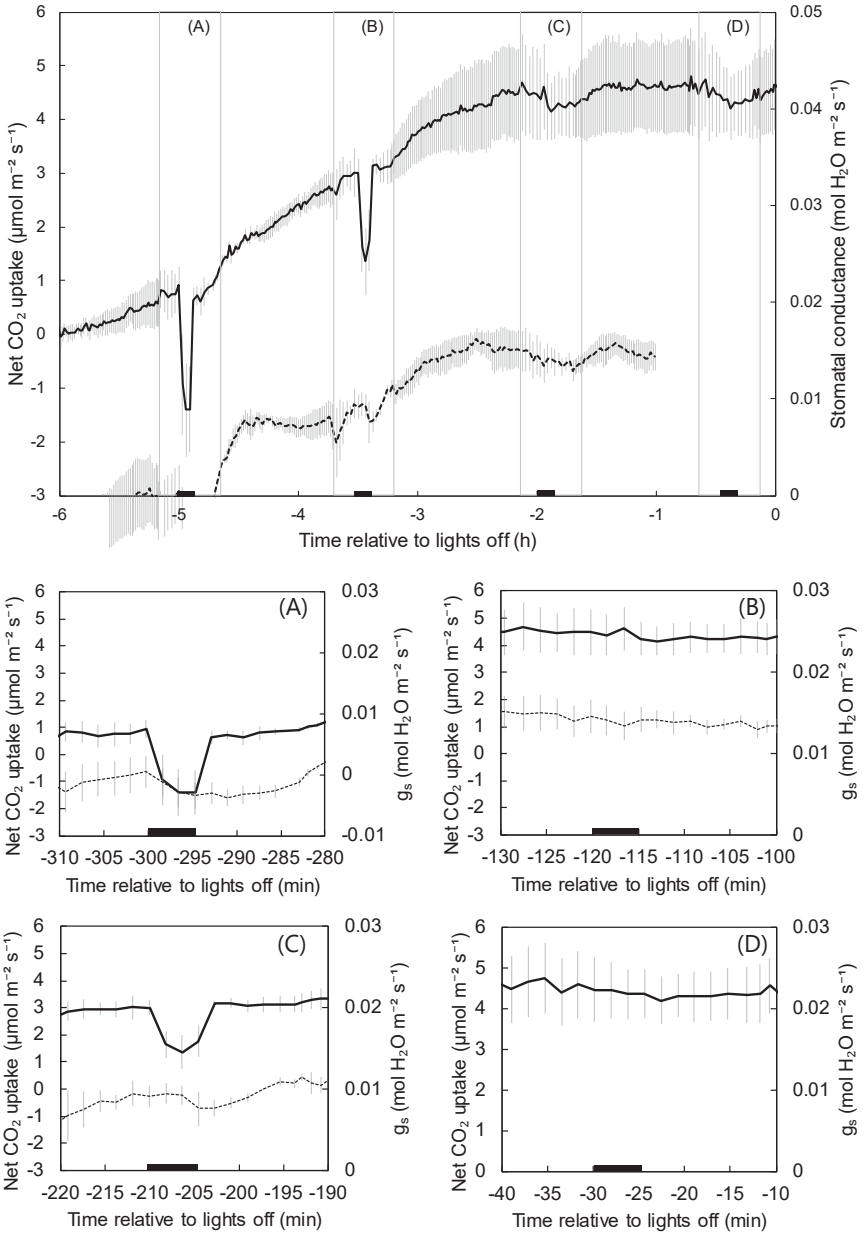

Figure 5.4 Response of CO_2 uptake (solid line) and stomatal conductance (g_s, dashed line) of *Phalaenopsis* leaves to a 5-minute dark period in phase IV. 5h (A), 3.5h (B), 2h (C) or 0.5h (D) before end of day, the light in the leaf chamber was switched off for 5 minutes before returning to 140 µmol photons m^{-2} s^{-1}, as indicated by solid black bar. Data represent the average of 3 replicates with SEM. g_s data from the last hour is missing due to irregularities in climate chamber humidity control. Plant growth conditions as described in Figure 5.1

exchange anymore, indicating full PEPC-carboxylation. A separate set of measurements with switching the lights off only at the last time point showed that lights off early in phase IV was not the reason CAM was induced at later time points (Supplemental information S5.1).

3.3. 2% O_2 to detect photorespiration during phase IV

In both species, switching to 2% O_2 resulted in an increase in CO_2 uptake and in a drop in ETR, meaning photorespiration occurs at all times in both species, except for *Phalaenopsis* at 225 minutes before lights off (Table 5.1), which is just after the start of phase IV. Here, ETR did decrease, although net CO_2 uptake was not different. The CO_2 uptake at this point was probably too low to measure differences. However, the change in ETR suggests Rubisco oxygenase activity behind closed stomata did occur. Switching to 2% O_2 resulted in an increased $NPQ_{(T)}$ in both species at all time points, but the relative increase in $NPQ_{(T)}$ was larger in *K. blossfeldiana* than in *Phalaenopsis*.

Table 5.1 Net CO_2 uptake rate (µmol CO_2 m^{-2} s^{-1}), electron transport rate (ETR) and non-photochemical quenching ($NPQ_{(T)}$) in young mature Phalaenopsis and Kalanchoe leaves at several time points (minutes relative to lights off) during phase IV in air with 21% O_2 or 2% O_2.

	Net CO_2 uptake			ETR			$NPQ_{(T)}$		
	Phalaenopsis								
Time before lights off	21% O_2	2% O_2	Sig.	21% O_2	2% O_2	Sig.	21% O_2	2% O_2	Sig.
-225	0.16 ±0.25	-0.03 ±0.41	NS	27.1 ±1.1	23.6 ±1.3	**	2.31 ±0.18	2.96 ±0.15	***
-150	2.23 ±0.41	3.34 ±0.51	***	21.8 ±1.2	18.8 ±1.2	***	3.15 ±0.17	3.58 ±0.18	***
-45	2.32 ±0.38	3.03 ±0.26	*	19.2 ±1.4	15.5 ±0.77	***	3.52 ±0.20	4.14 ±0.22	***
	Kalanchoe								
-150	0.42 ±0.16	0.84 ±0.17	**	38.1 ±1.1	33.9 ±1.5	***	0.82 ±0.12	1.32 ±0.26	*
-45	1.88 ±0.22	2.90 ±0.40	**	34.7 ±0.9	29.5 ±1.1	***	1.38 ±0.12	2.15 ±0.15	***

Data are means ± SE, measurements within one timepoint and within one species (n=8), Sig= significance. NS, *,**,*** are not significant, or significant at $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

3.4. Metabolite analysis

In *K. blossfeldiana* there was no accumulation of malate throughout phase IV (Figure 5.5A), suggesting that only C_3 photosynthesis occurred. There was some breakdown of citrate at the beginning of phase IV ($p=0.015$), although levels were generally very low and not considered

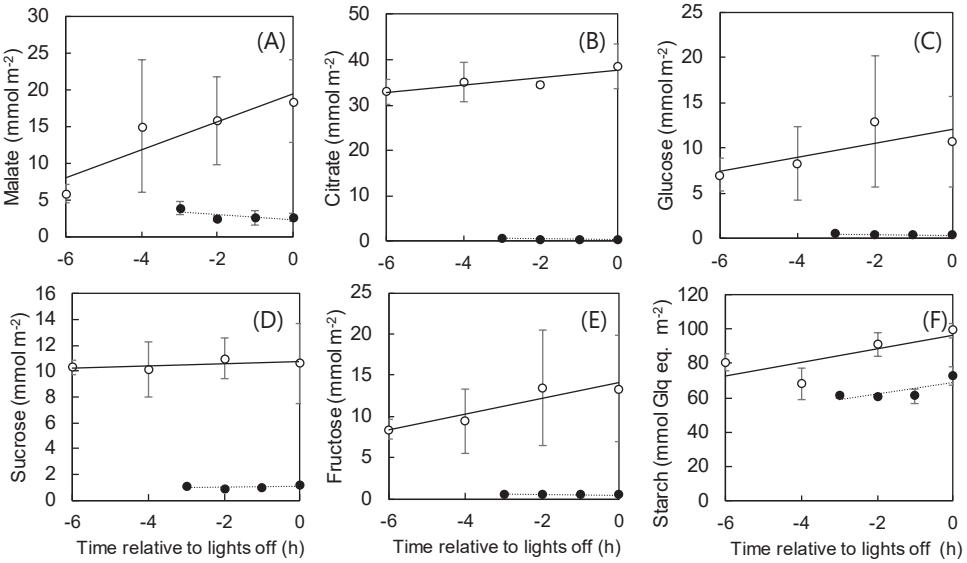

Figure 5.5 Levels of malate (A), citrate (B), glucose (C), sucrose (D), fructose (E) and starch (F) during Phase IV in leaves of *K. blossfeldiana* (closed circles) and *Phalaenopsis* (open circles). Data represent the average of 3 independent replicates with SEM bars. Plant growth conditions as described in Figure 5.1. See also Supplemental information S5.2 for results on malate and starch from a complementary experiment in *Phalaenopsis*. In this supporting dataset, the error bars are much smaller, and the malate concentration increased significantly in leaves of young vegetative *Phalaenopsis* plants during phase IV.

to contribute significantly to the CAM pathway. No significant changes during Phase IV were found for soluble sugars or starch in *K. blossfeldiana* (Figure 5.5C-F). *Phalaenopsis* showed some malate build-up, but differences between the beginning and end of phase IV were not significant due to large variation between samples ($p=0.159$). Data from a similar experiment on vegetative *Phalaenopsis* plants did show a significant build-up of malate in phase IV ($p=0.009$, see supplemental information S5.2). In this complementary dataset, sampling was done on a higher number of biological replicates with a sampling from the same leaf throughout the time of the experiment (one leaf, one replicate). This greatly reduced variance compared to the current dataset, and therefore showed a significant difference, which presumably could also be the case for the current dataset, if the same sampling methodology was used. There was no change in citrate levels in phase IV, nor in soluble sugars (Figure 5.5B-E). However, there was an accumulation of starch ($p=0.041$) in *Phalaenopsis* (Figure 5.5F).

4. Discussion

4.1. PEPC and not Rubisco is the main carboxylase in *Phalaenopsis*

In general, Rubisco is assumed to be the main carboxylase in CAM plants in phase IV (Osmond and Holtum, 1981). In C_3 plants, switching off the lights would result in a sharp drop in CO_2 uptake because light is needed to keep the Calvin-Benson cycle running and Rubisco carbamylated (Von Caemmerer and Edmondson, 1986). The CO_2 gas exchange profile measured in *K. blossfeldiana* confirmed that CO_2 uptake ceased when lights were switched off (Figure 5.1A). This is in line with previous studies on *K. daigremontiana* (e.g. Griffiths et al., 2002; Wyka and Lüttge, 2003) and *K. fedstchenkoi* (Borland et al., 2009) but also with studies on other CAM species such as *Mesembryanthemum crystallinum* (Dodd et al., 2003) *Clusia minor* (Grams and Thiel, 2002) and *Guzmania lingulata* (Maxwell et al., 1999). It is therefore not surprising that a similar gas exchange profile is shown in review papers on CAM plants (Lüttge, 2001, Borland et al., 2011, Winter, 2019).

Notably, a robust nocturnal CAM profile was maintained for both species, without applying a day-night difference in temperature. Optimum nocturnal temperature for CO_2 fixation in CAM plants typically is between 10-20°C (Yamori et al., 2014), and in general it is believed that either low night temperatures or a temperature differential are required for CAM to function (Buchanan-Bollig and Kluge, 1981, Nimmo, 2000). Additionally, we temporarily switched off the lights in the leaf chamber for five minutes at several moments throughout phase IV and continued to measure gas exchange. These blackout periods always resulted in substantial CO_2 release in *K. blossfeldiana*, indicating that Rubisco was the main carboxylase (Figure 5.3). Diel gas exchange profiles in *Phalaenopsis* showed a continued rate of CO_2 uptake without a drop when lights were switched off at the end of the day (Figure 5.1B). This is in line with gas exchange profiles measured by Lootens and Heursel (1998) in *Phalaenopsis* hybrids "70" and "L", but these were never placed in the context of carboxylation type in phase IV. In *Phalaenopsis*, blackout periods during phase IV showed a decrease in CO_2 uptake early on (Figure 5.4A). However, this no longer resulted in CO_2 release already during the second blackout. Later on, in phase IV (Figure 5.4C,D), the continued rates of CO_2 uptake suggested that PEPC was the primary carboxylase in *Phalaenopsis*, at a time when CO_2 uptake rates were

highest.

K. blossfeldiana retained a constant ΦPSII throughout phase IV until the end of the day, indicating that Rubisco activity did not decrease (Griffiths et al., 2008), which is in line with the CO_2 release measurements due to a short blackout. The chlorophyll fluorescence profiles for *Phalaenopsis* showed a gradual decrease of the photosystem II operating efficiency (ΦPSII) towards the end of the day, while the CO_2 uptake simultaneously increased (Figure 5.2). This indicated changes in the regulation and activity levels of both carboxylases, as a decreasing ΦPSII at steady irradiance can be directly linked to reduced Rubisco activity and increasing PEPC activity (Griffiths et al., 2008). These fluorescence data indicate that in *Phalaenopsis*, the up-regulation of PEPC occurs early in phase IV. This may mean that PEPC was the main carboxylase during phase IV, and not only at the end of the day, as was previously suggested (e.g. Osmond and Holtum, 1981; Borland and Taybi, 2004). These fluorescence data over phase IV are in full agreement with the effect of the blackout periods in phase IV on CO_2 exchange (Figure 5.3 and Figure 5.4). Differences in the response of CO_2 uptake, as well as in stomatal behaviour due to blackouts in phase IV (Figure 5.3 and Figure 5.4), suggest that two different pathways were indeed active in *K. blossfeldiana* and *Phalaenopsis*.

Metabolite control (Dever et al., 2015), degree of succulence (Von Caemmerer and Griffiths, 2009) and in particular levels of C_i may play a role in determining the stomatal conductance in CAM plants. The drawdown of C_i is linked to exhaustion of the supply of malate. This process is associated with stomatal opening, which marks the start of phase IV (Males and Griffiths, 2017). Bearing this in mind, the closure response of stomata after switching the lights off in *K. blossfeldiana* might be due to changes in C_i, which would not occur in *Phalaenopsis* when PEPC, carboxylating CO_2 independent of light, is active. For *K. daigremontiana*, it was found that Rubisco activity was highest early in phase IV, most closely resembling C_3 photosynthesis (Maxwell et al., 1999). By closing stomata in response to switching off the light, the response of *K. blossfeldiana* appeared indeed similar to what is seen in C_3 plants (e.g. Lawson and Blatt 2020). If C_i and C_c during phase IV in *K. blossfeldiana* would be very low and Rubisco highly active, this can lead to a high level of photorespiration (Maxwell et al., 1999, Borland et al., 2000). Carboxylation of CO_2 (in the form of HCO_3-) via PEPC is more effective in fixing CO_2 than Rubisco due to its higher affinity for CO_2 (Wyka and Lüttge, 2003), and because it does not induce photorespiration. This makes CO_2 uptake via PEPC at low C_i more efficient

than via Rubisco, even though this would come with a slightly higher energetic cost per CO_2 fixed (Shameer et al., 2018). CO_2 plays a role in the activation of Rubisco, higher ambient CO_2 can increase carbamylation of Rubisco in C_3 plants (Von Caemmerer and Edmondson, 1986). In CAM plants, this could result in an increased end of day fixation via Rubisco. It is likely that CO_2 uptake via Rubisco would be more favourable compared to PEPC at 600 ppm, due to its lower affinity Rubisco mediated CO_2 uptake would benefit more from increased [CO_2]. Furthermore, it could increase carbamylation status, which would further enhance the CO_2 uptake by Rubisco, but PEPC might still be the main carboxylase in *Phalaenopsis*.

4.2. Effect of light quality and day length on carboxylation activity in phase IV

Light quality can affect the functioning of CAM photosynthesis, although little research has been conducted in this area. Exposing *Phalaenopsis* plants to either narrow-band red or white light did not affect PEPC activity during phase IV (Zheng et al., 2019). In another recent study on *Phalaenopsis* 'Sacramento', the same cultivar as in the current study, the response for CO_2 uptake during phase IV was similar to ours, though we are using a more dominant red light (Hogewoning et al. 2021, in press). Obviously, growing plants under a narrow-band light affects photosynthesis and plant growth, regardless of species or photosynthetic type (e.g. Wang et al. 2009).

It is unclear to what degree the contribution of PEPC fixation in phase IV is influenced by an extension of natural day length, as results from previous studies have proven inconclusive (Sekizuka et al., 1995, Ceusters et al., 2010). For *Phalaenopsis amabilis,* a 16-hour day resulted in continuous CO_2 uptake from phase IV to phase I, suggesting fixation via PEPC at the end of the day. This was not the case for plants with a 12-hour day (Guo et al., 2012). Diel PEPC activity is regulated at transcriptional and posttranslational levels via the circadian clock through phosphorylation (Nimmo, 2000, Taybi, 2004, Hartwell, 2006). This reduces the sensitivity of PEPC to inhibition by malate, which enhances (nocturnal) CO_2 fixation. Boxall et al. (2017) showed that CO_2 fixation and malate accumulation occurred also without PEPC phosphorylation, albeit to a lesser extent. PEPC activity is highest during the night, but phosphorylation can already occur in phase IV, especially in a relatively longer light period (Borland and Taybi, 2004).

Lootens and Heursel (1998) showed a continuous CO_2 uptake from phase IV to phase I with a 12-hour day in vegetative *Phalaenopsis* hybrids "70" and "L", indicative of mainly PEPC-mediated uptake at the end of day in shorter days. A more recent study with a 12-hour day in *Phalaenopsis* hybrid 'Exquisite Edessa' showed a similar continuation of CO_2 uptake in darkness, combined with a decreasing ΦPSII towards the end of the day (Zheng et al., 2019). This showed that PEPC can play a large role in CO_2 fixation during phase IV in *Phalaenopsis*.

4.3. Futile cycling might serve as a mechanism to avoid photoinhibition

Under natural daylight conditions, Rubisco activity decreases towards the end of the day at lower light intensities, as carbamylation of active sites is light-dependent (Von Caemmerer and Edmondson, 1986). However, plants grown in a greenhouse with additional supplemental lighting, or in a climate chamber with constant light conditions until the end of the day, require a form of energy dissipation to prevent photoinhibition when Rubisco activity and C_3 carboxylation are downregulated. If Rubisco activity stays high, the Calvin-Benson cycle can function as an (indirect) electron sink to dissipate light energy (Griffiths et al., 2002). Carbon isotope discrimination data show that PEPC and Rubisco can be active at the same time (Ritz et al., 1986, Roberts et al., 1997), but this has so far been dismissed as playing only a small role in CO_2 uptake, as it is sub-optimal and increases chances of futile cycling (Griffiths et al., 1990, Maxwell et al., 1999, Winter et al., 2015).

Futile cycling is the concurrent production and degradation of malate, which can occur if both carboxylation enzymes are active at the same time (Dodd et al., 2002). Instead of malate (as malic acid) being stored in the vacuole after carboxylation via PEPC, it is immediately decarboxylated again into CO_2 and PEP via pyruvate (Osmond et al., 1996). This CO_2 might then be carboxylated again by PEPC, or it diffuses into the chloroplast where it is fixed by Rubisco, resulting in net carbon gain. When net CO_2 uptake occurred, the response of *Phalaenopsis* to 2% O_2 was similar to *K. blossfeldiana*, in the sense that CO_2 uptake increased, while ETR decreased (Table 1). These results indicate that photorespiration was occurring in both species. This makes sense in *K. blossfeldiana*, where Rubisco was the main carboxylase. In *Phalaenopsis*, there was also an increase in CO_2 uptake at 2% O_2, although this increase was less than in *K. blossfeldiana*. This indicated that Rubisco was also still active, alongside PEPC. The relative

increase in $NPQ_{(T)}$ when switching to 2% O_2 was much larger in *K. blossfeldiana* than in *Phalaenopsis*. Photon costs per mol CO_2 fixed for CAM are higher than for C_3 photosynthesis (Winter and Smith, 1996). With PEPC active, this explains why photochemistry in *Phalaenopsis* can dissipate more energy than in *K. blossfeldiana*, in a situation where CO_2 might be limiting. These results are in line with the build-up of starch in *Phalaenopsis* at the end of phase IV (Figure 5.5). In *Phalaenopsis*, the amount of net CO_2 uptake in the last two hours of phase IV, when PEPC was the main carboxylase based on switching off the lights (Figure 5.4), is well over the amount of malate accumulated over this period (Figure 5.5). This indicated that both carboxylases were probably active, and that futile cycling may be indeed occurring (Borland and Griffiths, 1997). While futile cycling is considered inefficient from an energy use perspective, it might help to maintain electron sink strength in phase IV, to dissipate absorbed light energy and thus to avoid photoinhibition while still contributing to net carbon gain.

5. Conclusion

With these results, we showed in several ways that PEPC can be the main carboxylase in *Phalaenopsis* 'Sacramento' in phase IV while Rubisco remained active throughout the whole phase as well. This contrasted with the findings in *K. blossfeldiana* 'Saja', and what is generally assumed for CAM plants. This conclusion is based on: (1) the continuous CO_2 uptake pattern at the end of the day in *Phalaenopsis* when the light was turned off, showing that CO_2 uptake was light-independent, whereas *K. blossfeldiana* showed a drop in CO_2 uptake rates. Temporarily switching off the lights while continuing to measure gas exchange showed that a decrease in CO_2 uptake occurred only at the beginning of phase IV in *Phalaenopsis* but not later on, whereas *K. blossfeldiana* showed a decrease in CO_2 uptake when lights were switched off at all time points in phase IV. The distinct response was confirmed by differences in patterns of stomatal opening from the same dataset. This was also confirmed by (2) a decrease in ΦPSII in *Phalaenopsis* throughout phase IV, indicative of a decreasing Rubisco activity while a steady ΦPSII was measured in *K. blossfeldiana*. (3) Measurements at 2% O_2 showed that photorespiration occurred throughout phase IV in both *Phalaenopsis* and *K. blossfeldiana*. Lastly, (4) accumulation of both malate and starch occurred in phase IV in *Phalaenopsis* but not in *K. blossfeldiana*. Combining the results of gas exchange, fluorescence and biochemical

analysis, we suggest that PEPC was the main carboxylase in *Phalaenopsis* in phase IV, even though Rubisco also remained active. This seems to lead to futile cycling because of double carboxylation but can help to avoid photoinhibition. Additional research, including e.g. online carbon isotope discrimination, would help to further strengthen these statements.

6. Acknowledgements

We are grateful for the help of Maarten Wassenaar on all gas exchange measurements, Jan Sommeijer for additional sample collection and analysis used in supplemental information S5.2, as well as with the help of Arjen van de Peppel on biochemical analysis.

Supplemental information S5

S5.1 Short blackout period procedure to distinguish C_3- and C_4-driven CO_2-uptake end of phase IV only

To make sure PEPC was not activated as a result of lights off during phase IV, measurements were also done on plants that only included the last measurement block in which the lights were switched off, 0.5h before end of day.

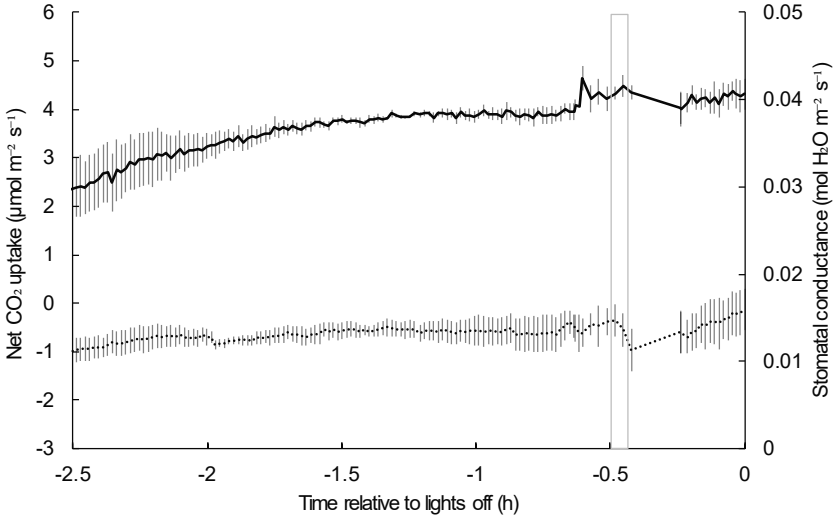

Figure S5.1. Late blackout period in *Phalaenopsis* cv. Sacramento. Response of CO_2 uptake (solid line) and stomatal conductance (dotted line) of young mature Phalaenopsis leaves to a blackout period 0.5 h before end of day. The light in the leaf chamber was switched off for 5 minutes during the period indicated by the grey outlined box, before returning to 140 µmol m^{-2} s^{-1}. This short blackout period procedure to distinguish C_3- and C_4-driven CO_2-uptake was done only once to determine whether there was an effect of earlier blackouts on plant response. Data represents the average of 3 measurements with SEM. For details on measurements and settings in leaf chamber during gas exchange, see material and methods under 'Short blackout period procedure to distinguish C_3- and C_4-driven CO_2-uptake during phase IV' (2.2.2).

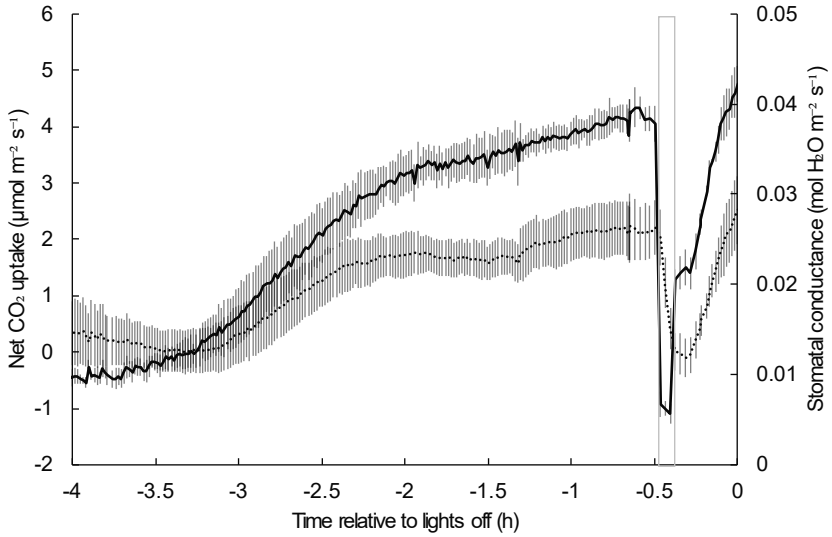

Figure S5.2 Late blackout period in *Kalanchoe blossfeldiana* cv. Saja. Response of CO_2 uptake (solid line) and stomatal conductance (dotted line) of third leaf pair from the apex to a 5 minute dark period in phase IV, 0.5 h before end of day. For method description see Figure S3.1.

S5.2 Diel patterns of starch and malate measured at two time points during vegetative growth in Phalaenopsis

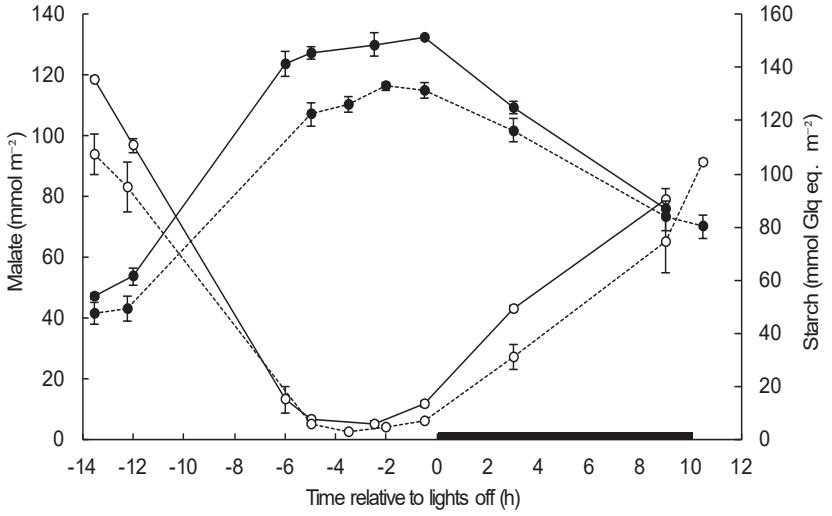

Figure S5.3 Diel starch (closed circles) and malate (open circles) pool size in young mature leaves of vegetative Phalaenopsis plants. Plants were grown in the greenhouse in 12 cm pots for 8 weeks after potting before placement in the climate chamber, where they were grown for 10 weeks (solid line) or 24 weeks (dashed line) at a temperature of 27°C and PPFD of 140 µmol m^{-2} s^{-1} with additional far-red of 23 µmol m^{-2} s^{-1} at a day length of 14 hours (Philips GreenPower LED production module deep red/white and GreenPower LED research module Far Red) at a photostationary state of 0.83 (Sager et al., 1988). CO_2 concentration was kept at 600 ppm and vapor deficit of the air at 7.2 g m^{-3}. Plants were watered every five days with nutrient solution that had an EC 1.2 mS cm^{-1} and pH of 5.7. For composition of the nutrient solution, see supplemental data (S2). Samples in time were taken from the same leaves (3 biological replicates, repeated 3 times) over 24 hours. Data represent the average of the 3 real replicates with SEM. Sampling in phase IV was done 5, 2.5, and 0.5 hours before lights off. These data did not show a significant accumulation of starch (p=0.108), but there was significant build-up of malate in phase IV (p=0.009).

Chapter 6
General discussion

Crassulacean acid metabolism (CAM) has independently evolved several times in different plant families and different climates, in areas all over the world (Edwards, 2019). It is a photosynthetic adaption focused mainly on stress survival (i.e. drought stress and/or high temperature)(Lüttge, 2004), and is characterized by the temporal separation of CO_2 uptake during the night and CO_2 refixation during the day, that occurs within one mesophyll cell (Figure 1.2). Refixation of CO_2 during the day occurs behind closed stomata, which results in increased water-use efficiency (WUE), compared to C_3 and C_4 plants (Borland et al., 2016). In a world that is subject to climate change, with increasing frequency of extreme weather events and extended periods of severe drought (Naumann et al., 2018), CAM plants contribute to diversification of agriculture on arid or semi-arid lands. Unfortunately, there is limited attention for CAM plants as an agricultural commodity, as they are generally not considered as highly productive (Lüttge, 2004). However, CAM plants might achieve commercially viable yields in semi-arid lands, especially when compared to growing C_3 or C_4 plants in the same area (Davis et al., 2015). Studying CAM from an agricultural perspective can help to further cultivate the potential of CAM plants. A way to study agricultural commodities is via the use of modelling. Modelling can help to increase knowledge, by improving the understanding of plant growth and developmental processes. Plant and crop models can give insight in how environmental conditions and/or management strategies, affect plant growth and development (Li et al., 2012). Currently, no mechanistic plant or crop models for CAM plants exist, because CAM plants are primarily studied as a photosynthetic adaption, and not as agricultural commodity.

In this thesis, I have used a set of *Phalaenopsis* hybrids as a case study, hereafter referred to by their genus name *Phalaenopsis* (see section 2.1 of chapter 1). *Phalaenopsis* is an economically important ornamental orchid that engages CAM. By using my proposed conceptual framework (chapter 2) as a guide, I aimed to increase understanding of physiology of CAM plants on different biological and temporal scales, ranging from CO_2 uptake on leaf level to biomass accumulation of the plant. I have studied the response of *Phalaenopsis* plants to temperature and light, linking diel processes to growth throughout the complete cultivation period. Experiments on a range of genotypes were conducted in climate chambers, where the environment could be accurately controlled. I have used 1) gas exchange to determine CO_2 uptake and to define the contribution and duration of CAM-phases within a diel cycle, 2) chlorophyll fluorescence to increase insight in to the functioning of the Calvin-Benson cycle

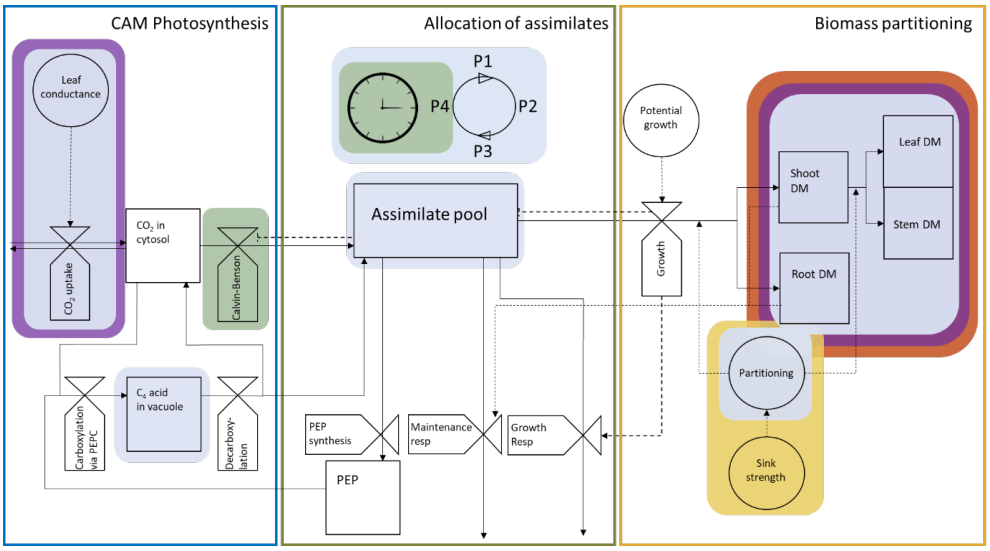

Figure 6.1 Conceptual framework for carbon fixation in crassulacean acid metabolism (CAM) plants divided into three modules: CAM Photosynthesis (1), Allocation of assimilates (2) and biomass partitioning (3)(Chapter 2). Forrester's (1961) symbols are used: boxes for state variables, valves for rate variables and circles for intermediate variables. Full-line arrows for carbon flows, and dashed-line arrows for information flows. Coloured boxes represent conducted measurements in chapter 3 (orange), chapter 4 (blue) chapter 5 (green) and supplemental information S6.2 (purple) and S6.3 (yellow).

and carboxylase type, 3) metabolite analysis to quantify the assimilate pool and storage of C_4 acids in the vacuole, and conducted destructive harvests to determine the effect on growth and development. Additionally, experiments were conducted to study the effect of red:far-red light and sink limitation in Phalaenopsis. For an overview of these measurements within the framework, see Figure 6.1.

In this general discussion I will reflect upon several concepts and conclusions of this thesis. First, I will review several considerations that are relevant when combining different temporal scales: linking diel measurements of both CO_2 (section 1) uptake and assimilate pool (section 2) to plant growth. Then, I will extend the discussion on the effect of environmental factors on *Phalaenopsis* beyond temperature and light intensity. I will propose how knowledge of this thesis could contribute to finding early selection criteria when phenotyping *Phalaenopsis* in breeding, and how increased knowledge of genotypic variation in *Phalaenopsis* can be useful for both breeders and growers (section 4). Additionally, I will focus on future research in CAM in general, discussing how the framework should be improved and what the implications are

for studying CAM plants in more temperate regions (section 5). Lastly, I end this discussion with some personal concluding remarks (section 6).

1. Linking CO_2 uptake to plant growth

1.1. Diel CO_2 uptake measured on a leaf is a good proxy for CO_2 uptake of the whole shoot

In chapter 4, I studied whether diel measurements (like CO_2 uptake and carbohydrate content) could help to explain differences in vegetative growth and development of two *Phalaenopsis* genotypes grown at a combination of different temperatures (27°C and 31°C) and light treatments (60 µmol m^{-2} s^{-1} and 140 µmol m^{-2} s^{-1}). Note that in commercial cultivation, the vegetative growth phase occurs at ±28°C, at a light intensity of ±100 µmol m^{-2} s^{-1}. Saturating light intensity for *Phalaenopsis* is believed to be in the range of 130-200 µmol m^{-2} s^{-1}, depending on temperature (Lootens and Heursel, 1998, Lee et al., 2019). The majority of CO_2 uptake occurs during CAM-phase I (see chapter 1 for an elaborate description of all CAM-phases). Phase I takes place during the night and is quintessentially CAM (Figure 1.1 and Figure 1.2). Cumulative CO_2 uptake was second highest during phase IV (at the end of the day, when stomata have reopened) (Figure 4.2). The contribution of phase II to total diel CO_2 uptake in *Phalaenopsis* was relatively low (<8%). At 140 µmol m^{-2} s^{-1}, phase II lasted longer than at 60 µmol m^{-2} s^{-1}, which resulted in an increased cumulative CO_2 uptake during this phase. This effect was larger at 27°C than at 31°C (Figure 4.2). At higher temperatures, the onset of malate release from the vacuole and subsequent decarboxylation starts earlier (Winter and Tenhunen, 1982).

Considering the heavy self-shading of *Phalaenopsis* plants, it was surprising to see the correlation between diel CO_2 uptake and plant biomass at the end of the vegetative growth phase (Figure 4.3). Mind that the correlation of CO_2 uptake and plant growth was only significant when CO_2 uptake was expressed on a per plant basis, not on a per unit leaf area basis (per m^2). Here, CO_2 uptake was always measured on the youngest mature leaf. This leaf receives the highest light intensity. Considering that *Phalaenopsis* is heavily self-shading due to its phyllotaxis of 180°, older leaves can intercept hardly any light, because light intensities

at lower leaf pairs are only a fraction of what the top leaves intercept. Therefore, the question remained whether a leaf measurement was a good representation of CO_2 uptake by the shoot as a whole. A whole-shoot chamber was built and in an additional experiment, diel CO_2 uptake measured on a leaf was compared to CO_2 uptake of a whole shoot (Supplemental data S6.1). I showed that, when expressed on a per unit leaf area basis cumulative diel CO_2 uptake measured using the shoot chamber was 89% of CO_2 uptake measured on a single leaf that receives most of the light. However, the contribution of different phases varied (Supplemental Table S6.1). These measurements were conducted on young vegetative *Phalaenopsis* plants, with 5-6 fully developed leaves. The relative difference between leaf and shoot chamber was largest in CAM-phase IV. In this phase, CO_2 uptake measured using the shoot chamber was much larger than what was measured using a leaf chamber. This was caused by a much later start of phase IV in the leaf chamber. It might be that older leaves started CO_2 uptake earlier than the youngest mature leaf, but this remains unclear and requires further study (see also section 1.6 of this chapter).

CAM plants do not take up CO_2 during phase III, as this is the period where decarboxylation of malate occurs behind closed stomata (see section 1.3 of chapter 1). Indeed, no CO_2 uptake was measured during phase III in the leaf chamber. However, the plant in the whole shoot chamber showed some CO_2 uptake had occurred during phase III. This is indicative of direct C_3 fixation, although the relative contribution to total diel CO_2 uptake was only small (3%). It is likely that CO_2 uptake during phase III was done by the youngest, not fully developed leaf. *Phalaenopsis* is an obligate CAM plant, but young tissue engages in C_3 photosynthesis and gradually shifts to CAM when maturing (Winter, 2019). It could be assumed that over time, the contribution of C_3/ CAM changes, which may affect biomass accumulation (Herrera, 2009), but this was not the case. From these results, I concluded that CO_2 uptake measured using a leaf chamber is a good proxy for CO_2 uptake by the whole shoot.

1.2. Causes of substantial CO_2 leakages during phase III

In chapter 4 I showed that a significant amount of CO_2 can leak out of the leaf during phase III in plants that were grown at 31 °C (Figure 4.2) This indicates that there might be a decoupling of biochemical sub-processes occurring at high temperatures, particularly when this is combined with a lower light intensity. The amount of CO_2 that will be fixed during the following

night is determined during phase III, but it is not exactly clear how this is regulated (Hogewoning et al., 2020). With an increase in light intensity, CO_2 uptake increases in all phases (except phase III where no net CO_2 uptake occurred)(Figure 4.3). Plants that were acclimated to a higher light intensity during phase III took up more CO_2 during the consecutive phase I than plants acclimated to lower light intensities (Figure 4.2) (Hogewoning et al., 2020). *Phalaenopsis* plants that were only exposed to high light intensity in the second half of phase III, had a much higher rate of malate breakdown compared to plants that were also subjected to high light in the first half of phase III (Hogewoning et al., 2020). However, the effect of light intensity on the decarboxylation rate of malate seems to be affected by other factors as well. Light intensity during phase III determines the rate of malate mobilization from the vacuole, which seems to be linked to electron transport rates (ETR) and the light-dependent assimilation of CO_2 via Rubisco (Lüttge, 2004).

Temperature affects fluidity and permeability of membranes in CAM plants, and an increase in temperature can enhance the rate of malate efflux from the vacuole (Friemert et al., 1988). Therefore, the tonoplast of CAM plants is very sensitive to acclimation to growth temperatures as it allows controlled malate accumulation and remobilisation during the CAM cycle, which is central to the functioning of CAM (Lüttge, 2004). Acclimation to higher temperatures leads to an increased expression of heat shock proteins. HSPs are important for e.g. protein folding, assembly and stability, and cellular homeostasis. HSPs also contribute to membrane stability (Wahid et al., 2007). In order to avoid CO_2 losses, an increased rate of malate efflux in response to increased temperature must also be accompanied by an increase in CO_2 fixation via Rubisco (Figure 1.2). Temperature acclimation could also lead to an increase of cyclic electron flow around PS_I, which could compensate for the increased ATP requirement (Yamori et al., 2014). Electron transport rates in CAM plants are already much higher than what is needed for CO_2 fixation (Griffiths et al., 2002) but especially at low light, this seemingly was not sufficient. It might be that, at low light and high temperature in phase III, electron transport capacity was limiting CO_2 fixation which lead such high pCO_2 that CO_2 leaked out of the leaf.

1.3. Linking CAM-phase IV to growth

Because of the assumed direct C_3 carboxylation in phase IV, this phase would therefore contribute most to growth, and thus increase productivity (e.g. Dodd et al., 2002; Nobel, 1996

and many others). However, Ceusters (2010) showed for the CAM-bromeliad *Aechmea* "Maya" that neither the amount of C_3/C_4 carboxylation, nor increased CO_2 uptake during phase IV could be linked directly to plant growth. How does this compare to *Phalaenopsis*? In chapter 4 I studied two genotypes, coded UPO4 and UPO6, and used PCA to show that there were clear differences in how they adapt to their environment and how they vary in processes ranging from CO_2 uptake to biomass accumulation, based on an in-depth analysis of parameters with the highest loading (Table 4.1). Here, I used data from chapter 4 to get an indication on the contribution of phase IV to total plant growth. This was done by expressing CO_2 uptake per plant during phase IV as a function of plant dry weight (Figure 4.2). CO_2 uptake in phase IV contributes 8%-23% to total cumulative CO_2 uptake. The extent to which phase IV correlated with plant biomass appeared to be strongly genotype dependent. In plants from UPO4, a positive correlation between CO_2 uptake in phase IV and plant dry weight was clearly visible (Figure 6.2A). This was not the case in plants from UPO6, where CO_2 uptake and plant biomass did not correlate (Figure 6.2B). The results presented in chapter 4 furthermore emphasize the importance of conducting experiments with sufficient genotypic variation, as stated previously, in particular in chapter 3. There was no clear effect of light intensity on the correlation of CO_2 uptake and plant growth. But, if CO_2 uptake in phase IV cannot be linked directly to plant biomass, does the carbon taken up during this phase contribute to growth and if so, how? These questions will be discussed in sections 2.1 and 2.2.

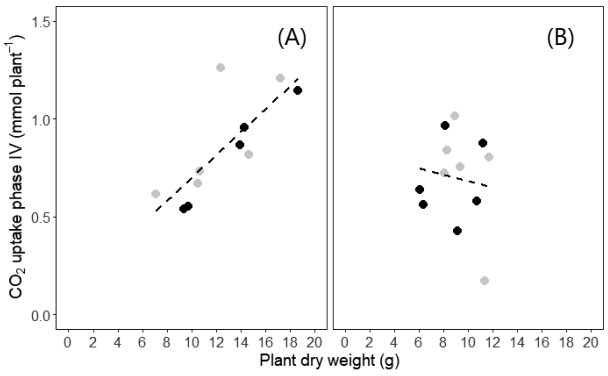

Figure 6.2 Cumulative CO_2 uptake in *Phalaenopsis* UPO4 (A) and UPO6 (B) during CAM-phase IV, expressed per plant as a function of plant dry weight, grown at PPFD of 60 µmol m^{-2} s^{-1} (black) or 140 µmol m^{-2} s^{-1}(grey). See chapter 4 for further details on plant material and growth conditions.

1.4. Carboxylase type during phase IV might depend on light intensity

For a very long time it was thought that of all CAM phases, phase IV most closely resembled C_3 fixation. Yet, in *Phalaenopsis* 'Saja', PEPC might be the main carboxylase in phase IV, and not Rubisco (Chapter 5). In chapter 5, but also in Hogewoning et al. (2021), we have discussed whether the fixation of CO_2 via PEPC during phase IV in *Phalaenopsis* could be caused by the extension of day length, something that is commonly done in commercial cultivation. That day length could affect the results presented in chapter 5 seemed plausible, as *Phalaenopsis* plants originate from an area where seasonality hardly occurs (Christenson, 2001) and where day length naturally does not extend beyond 12 hours. I reflected upon the matter in the discussion of chapter 5, where I mentioned several studies that showed that CO_2 fixation via PEPC also occurred in *Phalaenopsis* plants grown at a day length of 12 hours (Lootens and Heursel, 1998, Zheng et al., 2019). In CAM plants, both metabolite control and the circadian oscillator are responsible for coupling the phases (Dodd et al., 2002). In chapter 5, moderately high light levels (that is, moderately high for the shade plant *Phalaenopsis*) were given during the day (140 µmol m^{-2} s^{-1} for 16 hours per day). CAM plants usually grow in areas where light is abundant, and I wonder to what extent light intensity affects C_3/C_4 carboxylation in phase IV. Light intensity during phase III determines malate efflux and decarboxylation, and lower light intensities result in a longer phase III than higher light intensities (Hogewoning et al., 2020). If Rubisco activity is highest at the start of phase IV (Maxwell et al., 1999), it might be that the extended duration of phase III also results in CO_2 fixation via Rubisco in phase IV that lasts until later in (absolute) time.

Running the CAM cycle comes with an additional energetic cost (see chapter 1.1.2), and sufficient light is needed to support additional ATP demand. However, in studies where limited light is available in phase III (e.g. during winter and autumn in the work of Ceusters et al., 2010), several things could be happening. Firstly, in anticipation of expected light levels during the following day, nocturnal CO_2 fixation and subsequent malate accumulation is lower (Ceusters et al., 2010, Hogewoning et al., 2020), likely in order to balance allocation towards carbon required for CAM and for growth. Secondly, the ratio of C_3/C_4 carboxylation shifts towards more C_3 carboxylation in phase IV (Ceusters et al., 2010), possibly to further reduce ATP costs.

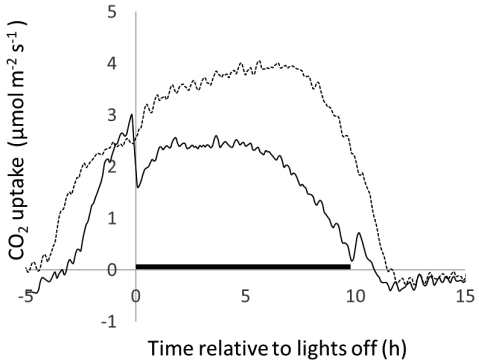

Figure 6.3 Representative curves of net CO_2 uptake of vegetative *Phalaenopsis*, grown at a PPFD of 60 µmol m^{-2} s^{-1} (solid line) or 140 µmol m^{-2} s^{-1}(dashed line). Solid bar on x-axis indicates night period. See chapter 4 for further details on plant material and growth conditions.

Indeed, a comparison of diel CO_2 uptake profiles of plants grown at 60 µmol m^{-2} s^{-1} and 140 µmol m^{-2} s^{-1} at 27°C showed that 1) phase IV started later in plants grown at 60 µmol m^{-2} s^{-1} compared to plants grown at 140 µmol m^{-2} s^{-1}, and 2) significant C_3 uptake seemed to occur at the end of the day at 60 µmol m^{-2} s^{-1}. This was visible as a drop in CO_2 uptake when lights were switched off. This did not happen in plants that were grown at 140 µmol m^{-2} s^{-1} (Figure 6.3). Both plants are grown at a day length of 14 hours. It would be interesting to see how light intensity, day length and subsequently, duration of phase IV determine the amount of C_3 /C_4 carboxylation during this phase.

1.5. Effect of the gas exchange methodology

Conducting gas exchange measurements with a device that is clamped onto the leaf such as the LI-6400XT is commonly done, but it can also induce some errors (Pons and Welschen, 2002). When logging CO_2 uptake and photosynthesis using gas exchange, only net CO_2 uptake is measured. This approach assumes that CO_2 exchange only occurs in the leaf chamber, and that there is no interaction with the rest of the plant outside of the leaf chamber, or interference of the leaf area under the gasket that is suddenly in darkness. The latter can cause overestimation of dark respiration (R_d), and this effect is relatively larger when CO_2 uptake rates are low (Pons and Welschen, 2002).

In CAM plants, clamping on a leaf chamber might have an additional effect. For the gas exchange measurements conducted in this thesis, the leaf chamber was clamped onto the leaf towards the end of phase III. The rate of malate efflux in phase II is affected by light intensity

(Lüttge, 2004) and clamping the leaf greatly reduces the light intensity for the leaf area in the leaf chamber and under the gasket. While light intensity within the leaf chamber is not homogeneous using the fluorescence head of a LI6400-XT to conduct measurements on gas exchange (Hogewoning et al., 2010), this statement is particularly true for the transparent leaf chamber. Light intensity within the transparent leaf chamber can be up to 30% lower compared to the light intensity directly outside of the leaf chamber, depending on how the leaf chamber is positioned on the leaf. This was considered during all measurements, as I made sure that, for plants belonging to the same light treatment, light intensity within the chamber was as similar as possible. The effect of reduced light intensity within the transparent chamber became apparent when conducting measurements over several days. CO_2 uptake during phase I continued to be lower in days following the first 24 hours (Figure S6.2). It is important to keep in mind that, if a treatment is applied on day 1 after start of the measurements under the assumption of a steady-state situation, the conclusion might be significantly affected by this error. Therefore, leaves of plants used in Supplemental experiment S6.2 (see the later section 2.3 of this chapter for a more detailed explanation of this experiment) were covered after steady-state was reached on day 4, and not on the first day. To avoid errors induced by data measured on different days, in all chapters, I have used data that was collected on day 1.

Due to clamping on the leaf chamber, the efflux of malate and its subsequent decarboxylation is reduced for the area under the gasket. It could therefore be a source of CO_2 that continues to exist into phase IV. It might then diffuse into the leaf area within the leaf chamber, interfering with gas exchange measurements. This could result in a lower measured net CO_2 uptake in the beginning of phase IV. The start of phase IV is defined as recurrence of net CO_2 uptake, meaning it could also result in a later apparent start of phase IV. The comparison of CO_2 uptake in *Phalaenopsis* measured using a leaf chamber to a whole-shoot chamber indicates that this might play a role. Cumulative CO_2 uptake in phase IV was much lower in the leaf chamber than in the shoot chamber (Table S6.1), which was the result of a later start of phase IV. However, if this would be due to clamping on the leaf chamber during phase III, it is expected that this deviation only occurs during the first phase IV, and not during subsequent days, but this was the case. It is therefore not likely that clamping on a leaf chamber caused this deviation between leaf and shoot CO_2 uptake, although it remains unclear what

the reason for this was. It might be that older leaves have re-opened stomata earlier than the youngest mature leaf, but this would require further study of CO_2 uptake by individual leaves.

It is important to be critical about the measurement setup that is used. However, a given methodology does not have to be flawless for it to be of use, as long as the user is aware of the effect it can have on the produced output. Because all measurements in this thesis were conducted in a similar way, this would make any error also similar. I therefore believe this has not affected the conclusions that are drawn here.

2. Linking the assimilate pool to plant growth

2.1. Quantifying carbon fluxes

The underlying processes contributing to the relation between CO_2 uptake and plant dry weight varied between genotypes and treatments (Chapter 4). While CO_2 uptake by a leaf could be linked to CO_2 uptake by the shoot, it did not seem possible to make a closed leaf carbon budget (Borland, 1996, Ceusters et al., 2010, Haider et al., 2012), when the assimilate pool was based on measuring soluble sugars, starch, and malate. Malate is stored in the vacuole as malic acid, which is the major C_4 acid for CO_2 storage in *Phalaenopsis*. Citrate does not fluctuate over a diel cycle in *Phalaenopsis* (Pollet et al., 2010, 2011). Respiration plays a central role in CAM plants (Tcherkez, 2017), but is particularly difficult to determine accurately, because respiratory CO_2 can also directly function as a source of CO_2. In this thesis, I have not quantified respiration fluxes of the shoot, and have not measured photosynthesis or respiration of the roots at all. Furthermore, I have not quantified any leaf exudates that were apparent on some plants (Figure 4.9) It might be that relevant metabolites have been missed in determining carbon flows, or that oversimplifications were made in the assumptions about steps in carbon processing. In order to quantify growth of *Phalaenopsis* based on carbohydrate content, more work is needed to identify and quantify possible fluxes of carbon.

2.2. Export of carbohydrates stored in phase IV might occur during subsequent phase I

I showed that all of carbon taken up in phase IV in *Phalaenopsis* is either stored in malate or in

starch (Figure 5.5). Malate that is stored in the vacuole will be broken down during the next day's phase III (Figure 1.2). However, the fate of starch is not equally clear. If starch is used during the following night as substrate for PEP, it could allow for an increased amount of CO_2 fixation. If so, starch that is stored during phase IV indirectly contributes to growth. However, to be able to increase productivity this must result in an increased malate pool that has more CO_2 stored, which in return would lead to more starch and so on, increasing growth in the long term. This implies that daily starch fluctuations in the leaf would increase over time during vegetative growth, but this was not the case (Supplemental information S5.3).

Borland et al. (2016) showed that degradation and export of starch (in the form of glucose or Glc6p) from the chloroplast to the cytosol can occur during the night (Borland et al., 2016), instead of directly during the day. Subsequently, this Glc6P can be converted to form PEP, or it can be used for export and growth in the form of sucrose. In order to have export of carbohydrates during the day, triose-P/P translocators (TP) have to be transporting triose-P from the chloroplast to the cytosol (Borland et al., 2016). If there is limited export of sucrose during phase IV, sucrose can accumulate in the cytosol, which can eventually block export of triose-P from the chloroplast. This will lead to the accumulation of 3-phosphoglycerate, which promotes starch synthesis (Paul and Foyer, 2001). Starch synthesis is further enhanced by the activity of glucose-6-phosphate transporter (GTP) which imports Glc6P into the chloroplast during the middle of the photoperiod in *Mesembryanthemum crystallinum* (Borland et al., 2016). If limited export can occur during the day, export for growth might take place later on and contribute to growth indirectly.

2.3. Effect of source-sink manipulation

The genotypes used in chapter 4 hardly showed an increase in plant dry weight with increased light intensity (Figure 4.1), whereas the average increase in plant dry weight of all genotypes used in chapter 3 was significant. However, genotypic variation was large in this study, and the increase in plant dry weight ranged from 5-44% (Figure 3.4). To rule out sink limitation as a cause for limited increase in growth at higher light levels in some genotypes, an additional experiment was set up (Supplemental experiment S6.2). While source strength depends on the amount of assimilates a plant can produce, sink strength depends on the ability of plant organs to attract assimilates (Marcelis, 1996). In a sink-limited situation, plants accumulate assimilates

(sugar and starch) because growth rates are lower than assimilate production. Accumulation of assimilates can serve as a feedback mechanism which can result in reduced rates of photosynthesis and accelerated leaf senescence (Paul and Foyer, 2001). I aimed to lower the source: sink ratio of young vegetative *Phalaenopsis* plants, by covering all leaves except the youngest mature leaf with black plastic. Doing so will reduce source availability, because covered leaves are no longer able to produce assimilates.

However, CO_2 uptake of the youngest mature leaf was not different between control plants that were (potentially) sink limited, and plants that were source limited (Figure S6.1). Interestingly, CO_2 uptake seemed to shift towards phase IV in source-limited plants, at the cost of phase II (Figure S6.2). However, variation was large, and combined with the limited number of replicates, this difference was not significant. A similar study on sink limitation in *Kalanchoe pinnata* showed that, while titratable acidity was reduced in the covered leaves, titratable acidity and sugar content of the uncovered leaves did not change (Mayoral et al., 1991). Although CO_2 uptake of the shaded leaves was not measured, the reduced, but still present, fluctuations in titratable acidity suggest that the shaded leaves might have switched to CAM cycling (Mayoral et al., 1991). On the short term, this would allow shaded leaves to maintain sufficient carbohydrate levels and not turn to sinks directly, as would be the case in C_3 or C_4 plants. Therefore, I cannot state with certainty that source: sink ratio was sufficiently altered to draw a definitive conclusion on sink limitation in vegetative *Phalaenopsis* plants.

3. Impact of environmental factors other than temperature and light intensity on Phalaenopsis

In this thesis I have focussed on the combination of temperature and light intensity, mainly during vegetative growth (Chapter 3 and chapter 4). However, other environmental factors, e.g. water availability, humidity, light spectrum and CO_2 concentration can affect growth and development in *Phalaenopsis* (e.g. Trouwborst et al., 2016*a*, Jin Kim et al., 2017, Lee et al., 2018). Studying and elaborating on all environmental factors was beyond the scope of this thesis, but I have studied the effect of red:far red (R:FR) ratio and CO_2 concentration in more detail, which will be expanded upon here.

3.1. Light spectrum

How light spectrum affects the expression and functioning of CAM is still unclear. Mechanisms that are relatively well understood in C_3 plants, such as the response of stomata to blue light, are not as straight forward in CAM plants, due to their reversed pattern of stomatal opening (Males and Griffiths, 2017). UV/A and blue light also seem to play a role in the induction of CAM. In the facultative CAM species *Clusia minor*, induction of CAM occurred only at specific wavelengths. When plants were exposed to light with wavelengths between 350 and 530 nm, nocturnal CO_2 uptake and daytime closure of stomata occurred, but not when plants were only exposed to wavelengths longer than 530 nm (Grams and Thiel, 2002). Light also affects expression of CAM in obligate species. Both light spectrum and light intensity seem to affect expression of CAM-related genes in *K. fedstchenkoi* (Zhang et al., 2020). Furthermore, light spectrum can affect synchronisation of processes essential to the functioning of CAM such as nocturnal CO_2 uptake, acidification and PEPC activity. Stomatal opening in phase II appeared to be induced by blue light and is reduced or completely absent when only red light was used (Ceusters et al., 2014). However, these results were observed when monochromatic light was applied. CO_2 uptake of plants that were grown under a combination of red and blue light was not significantly different from plants grown under white light, nor did they differ in malate content or PEPC activity (Zheng et al., 2019). Light spectrum does not only affect functioning and the expression of CAM, but affects plant growth and development in general. Relatively little research has been conducted on the effect of light spectrum on growth and flowering in *Phalaenopsis* (Runkle, 2019). Some studies indicate that light with a high R:FR ratio (i.e. high PSS) might reduce apical dominance and induce flowering in *Phalaenopsis* (Dueck et al., 2016). This could be beneficial in the cooling phase, when low temperatures are used to induce flowering (see chapter 2.2.1).

In this thesis I focussed on the effect of light intensity, rather than light spectrum. However, an additional experiment on the effect of different R:FR treatments showed that light spectrum can indeed affect flower development of *Phalaenopsis* (Supplemental information S6.2). I found no differences in CO_2 uptake or duration of CAM phases during vegetative growth between plants grown at different combinations of R:FR. However, in the flowering phase differences were apparent. While diel CO_2 uptake did not change, cumulative uptake in phase IV was lower whereas CO_2 uptake in phase I increased with additional far-red light,

compared to plants grown without additional far-red light. It remains unclear why this happened. More importantly, time to flowering was up to 10 days shorter with additional far-red, although the exact effect differed per genotype. This was surprising, as a higher amount of red light should reduce apical dominance and thus is more effective when inducing flowering (Lu et al., 2016). Here, the number of flower spikes was not affected, which was unexpected based on results of previous studies, where a high PSS resulted in a higher number of flowering plants (Lu et al., 2016) and higher number of flower spikes per plant (Dueck et al., 2016). It was surprising that such a high number of plants flowered in the study of Lu et al. (2016), because day/night temperature was set to 28/20°C. This should suppress flowering altogether, as it is temperature during the day, and not during the night that controls flowering in *Phalaenopsis* (Blanchard and Runkle, 2006).

In my study, the reduced time to flowering with lower R:FR was due to the combined effects of reduced time to flower induction and reduced time to flowering outgrowth. This contrasted with the results of Dueck et al., (2016), who showed that an increase in R:FR, rather than a decrease, enhanced flowering. While they studied the effect or R:FR in relation to temperature during the cooling phase, actual plant temperature data was not provided. In the experiment conducted in S6.3, exploratory measurements indicated that there were no differences in plant temperature that could be linked to R:FR ratio during the vegetative or cooling phase (data not shown). I applied treatments throughout the vegetative growth phase, not only during the cooling phase. It might be that applying additional far red during vegetative growth was key for the results that were found. In chapter 3 I showed that treatments (light intensity and temperature) applied in vegetative phase can have aftereffects during flowering. Both temperature and light can affect the carbohydrate status of *Phalaenopsis*, and increased carbohydrate content in the leaves can in return be linked to floral development (Chen et al., 1994, Kataoka et al., 2004, Hückstädt and Torre, 2013). Time to visible flower spike was positively correlated with carbohydrate content, in particular sucrose (Kataoka et al., 2004, Lee et al., 2019). It might very well be that, as shown recently for tomato fruits (Ji et al., 2020), low R:FR upregulates sink strength and sugar transportation to flower spikes in *Phalaenopsis*, which can have a beneficial effect during the cooling and flowering phases.

3.2. CO_2

In general, providing CAM plants with additional CO_2 was not considered very important as it is known from C_4 plants that PEPC saturates at ambient CO_2 (Winter and Engelbrecht, 1994). However, tightly packed mesophyll cells and low intercellular airspace can reduce diffusive supply of CO_2 and limit nocturnal assimilation (Males and Griffiths, 2017). During phase IV internal CO_2 can be as low as 100 ppm (Maxwell et al., 1999). Therefore, an increased CO_2 concentration of the air can very well result in an increased rate of CO_2 uptake in CAM plants. The effect of increased CO_2 on CAM appears to vary with species and per study. For *K. pinnata*, an increase in CO_2 concentration of the air resulted in an increased CO_2 assimilation rate, but it did not affect the carbohydrate pool for nocturnal provision of PEP-substrate (Winter and Engelbrecht, 1994). A substantial increase in diel carbon gain under elevated CO_2 was found for the CAM bromeliad *Aechmea* "Maya" (Ceusters et al., 2008), but only due to increased CO_2 uptake in phase II and phase IV. Furthermore, malate accumulation was hardly affected. It seems that CO_2 uptake via PEPC can only lead to enhanced carbon gain if several conditions are met (Hogewoning et al., 2020). In the case that CO_2 uptake during phase II and IV is mediated by PEPC instead of Rubisco, this might occur at the cost of nocturnal CO_2 storage, if the amount of PEP-substrate becomes a limiting factor in CO_2 fixation. Additionally, the vacuole must have sufficient capacity to store additional malate. If one or both conditions cannot be met, total CO_2 uptake would remain the same over 24 hours but with a shift away from phase I. This could result in a similar growth rate, but at reduced WUE. While this would be of minor concern in *Phalaenopsis* cultivation in greenhouses, it defeats the purpose of CAM in its primary functioning, as a water saving mechanism.

Providing *Phalaenopsis* plants with additional CO_2 does increase diel CO_2 uptake during vegetative growth and during flowering (Hogewoning et al., 2020). This study showed that neither vacuolar storage capacity for malate, nor the supply of substrate for CO_2 fixation (PEP) were limiting factors for carbon gain enhancement, as CO_2 uptake in phase I increased when plants that were grown at 400 ppm were exposed to 800 ppm. During vegetative growth the increase in CO_2 uptake in phase IV was not significant at 800 ppm compared to 400 ppm, but this was the case in the flowering phase. Despite the increase in CO_2 uptake, g_s was lower in plants measured at 800 ppm compared to 400 ppm (Hogewoning et al., 2020). The decrease in g_s at high CO_2 is found more often, and it is known that g_s is sensitive to changes in C_i (Wyka

et al., 2005). If increased ambient CO_2 can increase carbon gain in CAM plants, this might increase productivity. It would be interesting to see how this affects carboxylase type. Knowing when to provide increased CO_2 is helpful in making optimal use of resources. If additional CO_2 uptake during phases II and IV does not go at the cost of nocturnal fixation, this can be done without a decrease in WUE.

4. Perspectives for *Phalaenopsis* cultivation

4.1. CO_2 uptake during vegetative growth as a proxy for *Phalaenopsis* flowering quality

Growing *Phalaenopsis* is a global business, and large breeding companies aim to amaze and serve growers with their lush, exquisite plants that are grown for their flowers (Figure 1.4). Selection of new cultivars can take 10-15 years. From propagation in the lab until flowering can take up to two years, and this cycle must be repeated several times to determine plant performance in different seasons. Currently, no early selection criteria have been defined that would allow breeders to evaluate plant quality at an earlier stage, for instance during the vegetative growth phase. The lengthy vegetative phase is considered important, as it lays the foundation for a plant that can produce high quality flowers (Runkle, 2019). By increasing knowledge on ecophysiology and genetic variation, I aimed to find traits that could be measured during vegetative growth, and which are correlated with plant flowering quality. Breeding could be accelerated by finding selection criteria that allow breeders to discard low quality plants early, or by finding ways to increase rates of growth and development during vegetative growth.

In this thesis I showed that cumulative diel CO_2 uptake measured on a small leaf sample can serve as proxy for CO_2 uptake of the whole plant (Supplemental information S6.1). Diel CO_2 uptake was correlated with biomass accumulation in the vegetative phase, and could thus be used as a proxy for vegetative plant growth. Furthermore, vegetative plant growth can serve as an early indicator for flowering quality (Figure 6.4). Traits like number of leaves and biomass of the vegetative plant were a good proxy for, respectively, number of inflorescences and inflorescence biomass. The correlation of diel CO_2 uptake and plant growth holds when CO_2 uptake is expressed on a per-plant basis (Chapter 4), which requires knowledge on leaf area of

the plant. Here, this was done using destructive measurements, but leaf area can also be measured non-destructively, for instance by making use of plant cameras, potentially even in a high-throughput setting (Campbell and Nirman, 2018). It remains to be seen whether the correlation of CO_2 uptake and plant dry weight requires total leaf area of the plant measured, or if projected leaf area would be sufficient for this.

Ideally, leaf CO_2 should also be determined in such a way that clamping on to the leaf is not necessary. It avoids potentially damaging the plant, but more importantly, clamping on a device to determine CO_2 uptake requires manual labour, and automation is therefore not possible. Plant CO_2 uptake could be measured using a whole-plant chamber, like the aforementioned plant-camera setup. To what extent the aerial roots of *Phalaenopsis* would affect these measurements remains unclear, as they are capable of photosynthesis (Goh et al., 1983). It remains to be seen whether a point-measurement would be sufficient as indicator for the relation between CO_2 uptake and plant growth, or if this requires measurements on a specific CAM-phase, or even a complete diel cycle measurement.

Chlorophyll fluorescence might also be used as a proxy for plant growth, but this requires further study. Nevertheless, it can already be put to good use to gain knowledge on plant physiology. Chlorophyll fluorescence can provide insight into photosynthetic efficiency, carboxylation status and duration of different CAM-phases. In *Phalaenopsis* plants grown at a range of temperatures PSII efficiency showed a trend that was similar to what was found when CO_2 uptake was measured (Lee et al., 2020). Furthermore, a decreasing ΦPSII can indicate the shift from Rubisco to PEPC carboxylation (Figure 5.2), information that can be used to

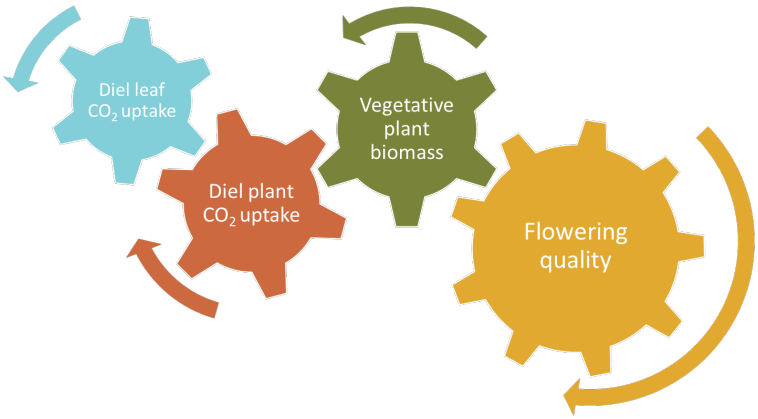

Figure 6.4 Schematic representation of the relation between key processes on different biological and temporal scales in *Phalaenopsis* breeding and cultivation as presented in this thesis.

determine strategies on supplemental lighting or CO_2. Chlorophyll fluorescence measurements can also be used to identify differences between *Phalaenopsis* genotypes (Ouzounis et al., 2015).

4.2. Knowledge of genotypic variation in Phalaenopsis is useful for breeders and growers

Genotypic variation in flowering traits is large in *Phalaenopsis*. Underlying mechanisms that contribute to growth and development also vary between genotypes. Knowledge on genotypic variation can be used to the breeders' advantage, because it can contribute to creating better plants. Insight in to underlying favourable traits that are not directly related to flowering quality, such as increased CO_2 uptake and WUE, can be used to breed plants that not only have high ornamental value but also high resource use efficiency. Knowledge on genotypic variation is very relevant for both breeders and growers. It allows breeders to provide better service when growers are choosing new cultivars, and can help growers to make well-informed decisions on e.g. the return on investment of supplemental lighting. Even more so, such knowledge can provide insight about during what times supplemental lighting is beneficial, or when it can cause light-stress (Hogewoning et al., 2020, chapter 5).

5. Considerations for future research on CAM

5.1. Improvement of the conceptual framework

The framework that is defined in chapter 2 can benefit from several improvements, if it would be used as a basis for quantitative modelling. Here, I will discuss two options to improve the framework, but it is important to note that these suggestions are not an exhaustive overview of all that is possible, and/or needed to mechanistically model growth and development of CAM plants.

Instead of using a framework or model where all similar organs are pooled, it could benefit from the modelling of individual organs, each parameterised based on developmental

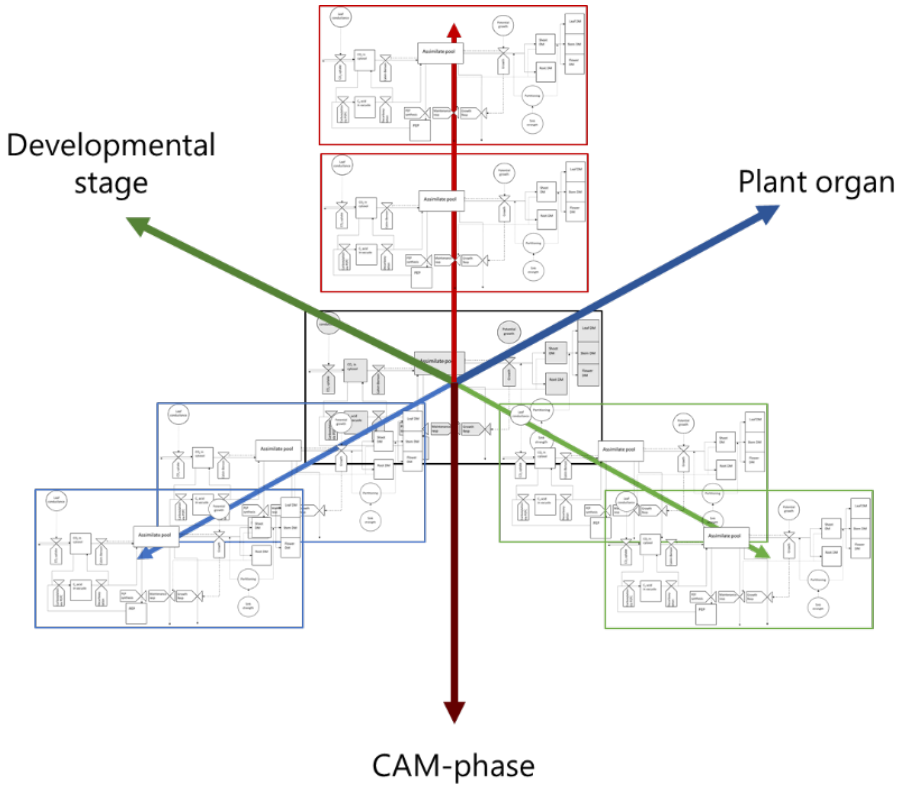

Figure 6.5 Schematic 3D representation of the conceptual framework to capture differences in developmental stage, CAM-phase and individual plant organs.

stage, microclimate, and CAM-phase (Figure 6.5). This is also useful for capturing the ontogenetic development that can exist throughout a CAM plant, or when the assumption of a homogeneous canopy does not hold. This is true for several CAM plants, such as *Phalaenopsis*, but also for *Agave* sp. and many members of the *Bromeliaceae* family. The within-plant effects may be captured by making use of a defining an elementary, repetitive unit within the model. These repetitive units, or building blocks, can be repeatedly created during the plants' development, but output can vary per unit, which allows for accurate modelling of e.g. light interception, leaf conductance, or assimilate allocation. This is commonly done in functional-structural plant modelling (FSPM)(Vos et al., 2010). This approach allows the combination of several integration levels within one model, while their basic structure is identical. For example, at lower levels, the building block can be a cell that builds up to be a tissue structure, whereas at a higher integration level, internodes can make up the building block. This approach can lead to a high level of detail within FSPMs, because it can eventually

lead to a multi-scalar model that functions at several levels of organization. It can be used to reach exceptional understanding of morphological development (Vos et al., 2010), although it does require collection of numerous data points.

Environmental conditions are currently placed as input outside the framework, but that means feedback caused by the environment is not captured within the framework. They should be included within the model. For example, transitional phases II and IV only occur if environmental conditions are favourable. As discussed previously, light and temperature affect the duration of phase III, which has implications for the other phases as well. Also the inclusion of a soil-plant-atmosphere-continuum (SPAC) model component, as is done by Bartlett et al. (2014) and consecutive iterations (i.e. Hartzell et al. 2015, 2018) in order to model the effect of soil dry down, shows the importance of having a model that interacts with the environment. The model was able to capture the shift from CO_2 uptake in phase I, II and IV all the way to respiration being the primary source of carbon (CAM-idling, see section 1.3 of chapter 1)(Bartlett et al., 2014).

It is important to learn from and incorporate other models that have been developed previously (e.g. Owen and Griffiths 2013, Hartzell et al. 2018, Niechayev et al. 2019, Töpfer et al. 2020, see also chapter 2 and references therein for a more complete overview), as most of them are much more elaborate and detailed in capturing CAM physiology than what I have attempted to describe in this thesis. However, these models do not mechanistically capture growth and development of CAM plants, which I believe is important for the development of CAM plants as an agricultural commodity and the main reason why this framework was developed.

5.2. Carbon isotope measurements can provide direct insight into carboxylase type

In this thesis, the methods that were used to determine which carboxylase was active during phase IV were indirect. I combined gas exchange data with short blackout periods, chlorophyll fluorescence and measurements at 21% and 2% O_2 (Chapter 5). The latter has been done before in order to determine carboxylase activity during the morning burst in phase II, when Rubisco and PEPC can be active simultaneously (Winter and Tenhunen, 1982). Chlorophyll fluorescence, when used to calculate ΦPSII, can be a good proxy for the amount of Rubisco/

PEPC activity (Griffiths et al., 2002). However, instantaneous carbon isotope discrimination measurements can be used to directly quantify which carboxylase is active and how CO_2 is fixed (e.g. Griffiths et al., 2002; Ritz, Kluge, & Veith, 1987; Roberts, Borland, & Griffiths, 1997). This might help with quantifying growth and calculating the energy budget of *Phalaenopsis* (and other CAM plants) based on the conceptual framework. Insight in which carboxylase is active can be used to optimise growing strategies, e.g. to determine when to provide supplemental lighting (Trouwborst et al., 2016*b*).

5.3. Studying the functioning of CAM in temperate regions

The questions raised earlier about CAM in an optimized production environment (Chapter 2), e.g. how phase II and IV contribute to growth and what determines maximum daily CO_2 uptake, are also relevant from a broader perspective. For example, when considering the bio-engineering of CAM into C_3 plants (see section 1.4 of chapter 1). C_3 plants usually grow in a more temperate climate, where CAM was never particularly favourable for survival or productivity (Cushman, 2017). However, as increasing frequency of drought and rising temperatures cause water-supply demands to rise significantly (Naumann et al., 2018), calls for alternative solutions to feed the world increase. Recently, Töpfer et al. (2020) showed via modelling that growing CAM plants in a temperate climate can still significantly reduce water use within a diel cycle. Interestingly, their model pointed out that carbon fixation via (iso)citrate dehydrogenase (ICDH, a mitochondrial enzyme that is part of the tricarboxylic acid cycle (TCA) or Krebs cycle) has an additional water-saving effect, up to almost 11%. Plants have either high citrate or isocitrate levels, but not both (Chen et al., 2002). However, neither fluctuated over a diel cycle in *Phalaenopsis* 'Edessa' (Ceusters et al., 2019). The contribution of citrate as a storage acid varies per species (Lüttge, 1988). While vacuolar capacity was limiting the functioning of CAM in the model of Töpfer et al. (2020), running the model with a C_3 leaf anatomy (i.e. vacuole size, leaf thickness and leaf porosity of an average C_3 leaf) already reduced water use by almost 20%. Running a partial CAM cycle can reduce water use within a diel cycle by more than 50%, while 80% of productivity is maintained. However, they do note that this is true only when assuming an average CAM-leaf morphology (i.e. with increased vacuolar volume per unit leaf). Strong CAM is associated with increased succulence of photosynthetic tissue (Winter et al., 2015). The modelling results of Töpfer et al., (2020) contribute to the discussion on why

bioengineering CAM into C_3 plants could be very beneficial, as it can greatly improve WUE (Yang et al., 2015, Winter, 2019).

6. Personal concluding remark

Phalaenopsis is an economically important horticultural crop that utilizes CAM photosynthesis. While *Phalaenopsis* is not your typical model-CAM plant, the contributions of commercial companies involved in *Phalaenopsis* breeding, lighting and consultancy gave me the wonderful opportunity to undertake this study on CAM. I aimed to increase insight in the functioning of CAM, by linking processes on different biological and temporal scales. The type of physiological research that I have conducted, studying a range of genotypes in a growth chamber that allowed me to accurately control the environment, has provided useful insights for cultivation of *Phalaenopsis,* and the way *Phalaenopsis* plants engage CAM. The knowledge produced in this thesis can be utilised by both breeders and growers of *Phalaenopsis*. Additionally, it should also be seen as a step-up for future CAM research, whether that involves *Phalaenopsis*, or another species.

From time to time, it has been difficult to balance between the broader perspective on the impact and position that CAM plants (can) have in agriculture, with making the results relevant and timely for *Phalaenopsis* cultivation. While conducting this research, I tried to keep in mind that the way plants are grown in The Netherlands, in a high-tech and highly controlled environment, is very different from agriculture in most parts of the world. It sometimes felt like working on one CAM species is a niche within a niche, and when that's a species that's grown within a greenhouse, even more so. Additionally, the wide range of habitats that harbour CAM plants make it difficult to generalize statements on the physiology of CAM plants anyway. When it comes to photosynthesis research, plant scientists always seem to be looking for harder, better, faster, stronger. And oh, preferably in a C_3 or C_4 crop. Yes, there lies much potential in, for example, bio-engineering CAM into C_3. However, this focus does draw attention away from the potential that CAM plants naturally have, about which there is still so much to learn. The work that is presented here on *Phalaenopsis* is an example of that. I strongly believe that CAM plants, as they are, are a part of the solution in creating a sustainable, climate-resilient and future-proof agriculture. I hope that this thesis can contribute to that future, if only in the slightest way.

Supplemental information S6

S6.1 Comparison of diel CO_2 uptake measured using a leaf chamber compared to a whole-shoot chamber[1]

The big-leaf approach that is commonly used to calculate the carbon balance of a plant assumes that a whole plant, or even a crop, has the same relative response as a young mature, unshaded leaf in the top of the plant. In doing so, the plant is treated as one homogeneous entity (Sprintsin and Chen, 2009). The heavy self-shading of *Phalaenopsis*, as well as the ontogenetic development of CAM with maturing tissue throughout a CAM plant (Winter, 2019) can lead to an overestimation of plant CO_2 uptake or a wrongful assumption on the contribution of different CAM-phases (Osmond, 1978). Furthermore, clamping on a leaf chamber might introduce additional errors (Pons and Welschen, 2002). Therefore, I tested whether diel leaf CO_2 uptake measured with a leaf chamber (LI-6400XT) is a good proxy for whole shoot photosynthesis.

Material and methods

Young vegetative *Phalaenopsis* plants of genotype UPO19 (with 4-6 developed leaves) were grown in a Venlo type glasshouse (Bleiswijk, The Netherlands) in plugs, before they were placed inside a growth chamber. Temperature was set to 27°C, vapor pressure deficit of the air was set at 1 kPa. Plants were illuminated for 13 hours at a PPFD of 105 (±3) µmol m^{-2} s^{-1} and additional far red of 30 (±5) µmol m^{-2} s^{-1} (Philips LED production module deep red/white and GreenPower LED research module Far Red; Signify, Eindhoven, The Netherlands). Here, red light is defined as light between 600 and 700 nm, and far-red as light between 700-800 nm. This resulted in a R:FR ratio of 3.4 (±0.7), which corresponds to an estimated PSS of 0.82. Plants were watered 5 hours before the start of the measurements. Leaf gas exchange measurements were done using LI-6400XT (Li-Cor Inc., Lincoln, USA), with a flow rate of 150 µmol m^{-2} s^{-1}, on the youngest mature leaf. Humidity was set to 55% at a block temperature of 27°C. Matching was done every 4 minutes and data was logged every 20 seconds. A custom made whole-shoot

[1] MSc student Seppe Salari has participated in the development of the shoot chamber, alongside Maarten Wassenaar and Wim van Ieperen. Seppe has collected and analysed the data that is shown here.

chamber was used to determine diel CO_2 uptake of the shoot (n=3). Setpoints in the plant chamber were similar to those in the climate chamber with a CO_2 concentration of 600 ppm and humidity of 55%. Flow-rate through the shoot chamber (of 15L) was set to 7 L min^{-1}. Light intensity within the shoot chamber was 105 µmol m^{-2} s^{-1}. Leaf area of the plants was measured (Li-3100, Li-Cor Inc., Lincoln, USA) and used to calculate the gas exchange per unit of leaf area (m^2), in order to compare to the leaf chamber results. Results are the average of 6 consecutive days of measurements.

Results

The average leaf area of the shoot was 0.03 m^2, which was used to calculate CO_2 uptake per m^2 leaf area. CO_2 uptake measured in the leaf chamber and shoot chamber were in the same order of magnitude when they were both expressed on a leaf area basis (Table 1). CO_2 uptake of the whole shoot was 89% of CO_2 uptake measured with the leaf chamber. Cumulative CO_2 uptake in phase I and IV combined accounted for the majority of diel CO_2 fixation. The relative difference between leaf and shoot chamber was largest in phase IV. In phase III, CO_2 uptake in the leaf chamber was slightly negative (but close to 0%), whereas CO_2 uptake during phase III in the plant chamber accounted for 4% of total diel CO_2 uptake.

Table S6.1 Cumulative CO_2 uptake (mmol m^{-2}) of young vegetative *Phalaenopsis* plants expressed per CAM-phase and over 24 hours. Relative contribution of each phase is expressed between parentheses. CO_2 uptake was measured of the whole shoot using a plant chamber (n=3), or of the youngest mature leaf using a leaf chamber of LI-6400XT (data of a representative measurement

	Phase I	Phase II	Phase III	Phase IV	24 hours
Shoot	60.9 (65%)	6.8 (7%)	3.5 (4%)	22.9 (24%)	94.0
Leaf	89.2 (85%)	5.7 (5%)	-0.38 (0%)	11.39 (10%)	105.8

S6.2 Sink limitation in *Phalaenopsis*[2]

Plant biomass hardly increased with increased light intensity in genotypes used in chapter 4 (Figure 4.1), while the large set of genotypes used in chapter 3 did show an increase in plant

[2] Data shown here were collected and analysed by MSc student Stamatios Kottas.

biomass. Therefore, I determined whether sink limitation might play a role in limiting CO_2 uptake and plant growth in young vegetative plants of some *Phalaenopsis* genotypes.

Material and methods

Plant material and climate chamber settings were as described in S6.1, but at a PPFD of 98 ±3 µmol m^{-2} s^{-1}. All leaves except the youngest mature leaf were covered with black plastic. In some occasions, the youngest top mature leaf was followed by an immature small sized leaf, which was considered as a sink and for that reason it was also not shaded during the experiment. Material used for shading blocked 99% of light. For all measurements, the youngest mature leaf was used. Gas exchange measurements were done using LI-6400XT (Li-Cor Inc., Lincoln) with a transparent chamber (6 cm^2). To achieve sufficient air mixing and to dampen fluctuations in CO_2 concentration of the air, a buffer volume of 1000L with a fan was connected to the air inlet. Data was logged every 20 seconds. Each measurement of the experiment required the monitoring of 6 consecutive days after the first day of clamping on the LI-6400XT. Control and treatment data were collected simultaneously (n=4).

Results

In order to reduce variation between measurements in the analysis, data were normalised relative to measurement day two, when steady state conditions were assumed. Cumulative diel CO_2 uptake did not vary between treatments (Figure S6.1). However, the timing of CAM-phases of the plants with leaves covered with plastic shifted, compared to the control plants. CO_2 uptake in these plants seemed to shift towards increased CO_2 uptake in phase IV, and decreased CO_2 uptake in phase II (Figure S6.2). However, because of large variation, these differences were not significant.

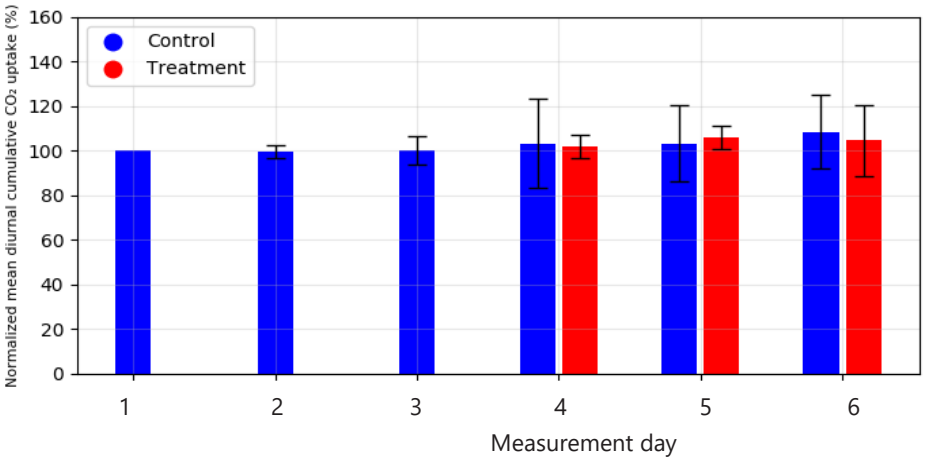

Figure S6.1 Cumulative CO_2 uptake (±95% CI), of young mature *Phalaenopsis* plants. In treatment plants, all mature leaves except the youngest mature leaf were blacked out with a black plastic cover. Data is normalized relative to day 1.

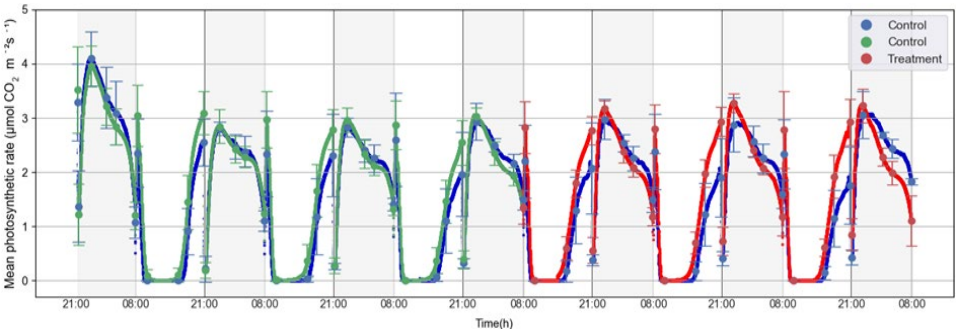

Figure S6.2 CO_2 uptake of the youngest mature leaf (±95% CI). Profiles are separated into leaves of control plant (blue curve) and leaves of plant that was otherwise completely blacked out (green for the days before shading application and red for the days after other leaves were blacked out). In treatment plants, all mature leaves except the youngest mature leaf were covered with black plastic.

S6.3 Effect of red and far-red light on diel CO_2 uptake and plant growth in *Phalaenopsis*[3]

Besides the effect of light intensity, I have also studied the effect of red:far-red ratio on gas exchange and plant growth and development, ranging from vegetative growth to flowering plants.

[3] Data shown here have been collected by MSc students Stefan van den Boogaart and Kuo Chen

Material and methods

Vegetative *Phalaenopsis* plants of six genotypes were grown for 14 weeks in a Venlo type glasshouse (Bleiswijk, The Netherlands) in 12 cm transparent pots filled with coconut bark, before they were transferred to a climate chamber at a plant density of 36 plants m^{-2}. CO_2 was set to 600 ppm and vapor pressure deficit of the air to 1 kPa. Plants were grown vegetatively for 10 weeks at 27°C, before a cooling phase of 6 weeks at 19°C induced flowering.

Subsequently, temperature was set to 22°C to promote flower development and outgrowth. Plants were illuminated for 14 hours per day by red/white, and far-red LED modules (Philips LED production module deep red/white and GreenPower LED research module Far Red; Signify, Eindhoven, The Netherlands) at a PPFD of 140 µmol m^{-2} s^{-1}. Light treatments consisted of different amounts of far-red light (n=4), with 97, 22 and 2 µmol m^{-2} s^{-1} resulting in PSS values of 0.72, 0.83 and 0.88, respectively (Figure S6.3)(Sager et al., 1988). Gas exchange measurements were conducted on genotype UPO2, during vegetative and flowering stage similar as described in chapter 4, but with block temperatures matching climate conditions (27°C and 22°C, respectively). During the flowering phase, time to consumer-ready stage (see chapter 3) was logged, counted from start of flower induction (cooling phase). Destructive measurements were conducted at the end of each phase as described in chapter 3. CO_2 uptake data were analysed using linear mixed-effect models using R version 3.6.1 (R Core Team, 2019) with package lme4 (Bates et al., 2015). As this experiment was conducted using two climate chambers, chamber was included as random effect.

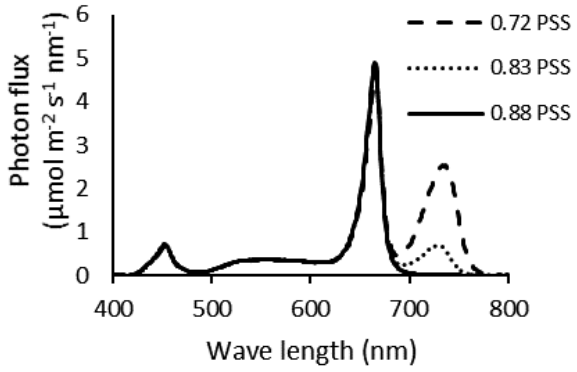

Figure S6.3 Representative spectral distribution of light treatments.

Results

During vegetative growth, no differences ($p<0.05$) were found in CO_2 uptake between treatments. This was true for both CO_2 uptake per phase, and cumulative CO_2 uptake over 24 hours. CO_2 uptake during flowering was affected in phase IV ($p=0.02$), phase I ($p=0.04$), and phase II ($p=0.005$). However, because CO_2 uptake was lowest in phase IV at a PSS of 0.72 but highest during phase I (and the opposite at PSS 0.88), no differences were found in cumulative CO_2 uptake over 24 hours (Figure S6.4).

Plant growth and development was not different during vegetative growth in response to treatments. However, from start of the cooling phase to consumer-ready stage, differences were apparent. A lower PSS reduced time to flowering up to 10 days for plants from the same genotype (Figure S6.5A). This did not affect the number of flower spikes that were formed. Number of flower spike was not affected by PSS (Figure S6.5B).

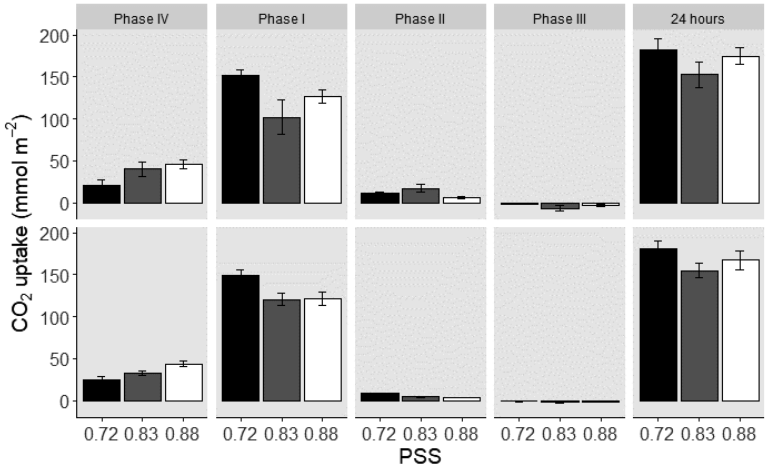

Figure S6.4 Cumulative CO_2 uptake of *Phalaenopsis* plants of genotype UPO2, expressed per phase and per diel cycle during the vegetative (top) and flowering (bottom) phase. Plants were grown at PSS of 0.72 (black), 0.83 (grey) or 0.88 (white). Data represent averages (n=4, except for plants from vegetative phase at PSS 0.72, where n=3) ±SEM error bars. CAM-phases expressed according to Osmond (1978).

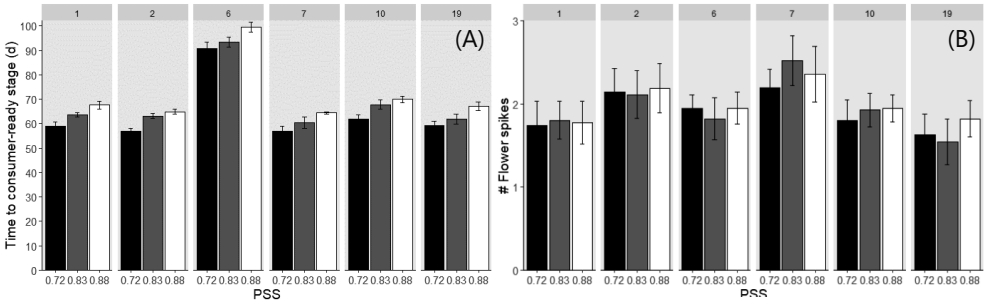

Figure S6.5 Time to consumer-ready stage (A) and number of flower spikes at the consumer-ready stage (B) of 6 *Phalaenopsis* genotypes exposed to light with different PSS values, counted from the start of the cooling phase. Data represent averages ± SEM error bars. Different numbers refer to different genotypes, of which numbers correspond with coding of genotypes used in chapter 3 and 4. For detailed phenotypic information, see supplemental information S3.1.

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Summary

Crassulacean acid metabolism (CAM) is a photosynthetic adaption that has evolved in response to water stress and/ or high temperatures. CAM can be found in plants that grow in (semi)-arid habitats, but CAM also appears in plants in the humid tropics, for example, in epiphytes that have no direct access to soil. CAM plants are remarkable at conserving water, because they take up CO_2 during the night, which allows them to keep their stomata closed during the hottest part of the day. CAM is usually not considered a favourable trait in agriculture, because CAM plants are (wrongfully) seen as not very productive. Therefore, relatively little research has been conducted on CAM plants, compared to C_3 and C_4 plants. The aim of this thesis was to increase understanding of CAM physiology by studying the economically important ornamental orchid *Phalaenopsis*. To reach this objective, *Phalaenopsis* plants were grown at several combinations of temperature and light. The response to these treatments was determined on different biological scales (from leaf to plant growth) and on different temporal scales (from seconds to the entire period of cultivation).

Chapter 1 introduces CAM and describes how the diel cycle of CO_2 uptake and CO_2 refixation can be captured in four phases. CAM comes with additional energetic costs, which are described in this chapter. Furthermore, the spectrum of phenotypic variation in CAM plants is synthesized. CAM plants are often not considered when looking for future-proof solutions in agriculture, but one option that is being explored is the bio-engineering of CAM into C_3 plants, which is also discussed here. The second half of this general introduction focuses on CAM in the orchid *Phalaenopsis*. The cultivation practice of the economically important *Phalaenopsis* pot plant is outlined, and the effects of temperature, light and CO_2 on *Phalaenopsis* growth and development are summarized.

Chapter 2 introduces a conceptual framework for CAM plants. A synthesis of the status of current CAM models revealed that currently no mechanistic models for CAM growth exist. Therefore, a conceptual framework was developed, which consists of three modules and combines processes related to 1) CAM photosynthesis, 2) allocation of assimilates among carbon pools, and 3) biomass accumulation among plant organs. The framework covers processes related to CAM physiology on different biological (from leaf to plant) and temporal (from hours to a full cultivation period) scales. This framework can function as the basis for development of a mechanistic crop growth model for CAM, but it can also help to identify

knowledge gaps that are present in the understanding of CAM. Development of the framework showed that it is still unclear how carbon is processed and allocated within CAM plants, when diel carbon fluxes are linked to plant growth. Implications of studying CAM in a non-limiting environment are also discussed. In this thesis, the conceptual framework served as the foundation for the other chapters, as it gave direction to which processes were relevant to study in order to increase understanding of CAM in *Phalaenopsis*.

Chapter 3 demonstrates that genotypic variation is large between *Phalaenopsis* hybrids. In two experiments with 19 and 14 genotypes, the effects of temperature and light on vegetative growth traits were studied. Furthermore, the after-effects of these treatments on flowering traits were determined. Hierarchal component analysis provided insight in how traits correlated and how they contributed to growth and development of *Phalaenopsis*. Plants that were grown at 31°C showed a strong reduction of plant and root growth (biomass accumulation) compared to plants grown at 27°C, while leaf initiation rates increased. An increase in light intensity (from 60 µmol m^{-2} s^{-1} to 140 µmol m^{-2} s^{-1}) accelerated both vegetative plant growth (biomass accumulation) and development (leaf initiation). Both an increase in light intensity, as well as an increase in temperature during the vegetative phase resulted in an increased number of flower spikes, and number of flowers and buds. In this chapter it is demonstrated that vegetative plant traits can be used to predict flowering quality despite genotypic and phenotypic variation. Traits like number of leaves and biomass of the vegetative plant are a good proxy for, respectively, number of inflorescences and inflorescence biomass.

Chapter 4 describes physiological differences between genotypes that underpin plant growth and development of *Phalaenopsis*. Plants from two genotypes were exposed to light and temperature treatments comparable to those in chapter 3. Measurements on gas exchange and carbohydrate analysis within a diel CAM-cycle were combined with measurements of plant growth and development. Per genotype, principal component analysis (PCA) was used to identify which traits explained most of the variation that occurred. Genotypes were found to differ in processes related to all three modules of the framework described in chapter 2. The two genotypes varied in the duration of CAM-phases, transpiration and CO_2 uptake rates. The two genotypes showed a contrasting response to environmental conditions in the amount of starch that accumulated during the day, and was broken down during the night, as well as in root biomass, and number and thickness of leaves. This chapter elucidated that a correlation

exists between cumulative diel CO_2 uptake and vegetative plant dry weight of *Phalaenopsis*, but only when leaf area of the plant is considered, not when expressed on a m^2 basis.

Phase IV is considered important for its substantial contribution to CO_2 uptake and to the productivity of CAM plants. Therefore, chapter 5 examines CO_2 uptake via C_3 and C_4 carboxylation in phase IV in the CAM species *Phalaenopsis* 'Sacramento' and *Kalanchoe blossfeldiana* 'Saja'. Short blackout periods, switching to 2% O_2 and measurements of chlorophyll fluorescence during phase IV all indicated that in *Phalaenopsis* 'Sacramento', PEPC might be the main carboxylase. This is unlike what is known from other CAM species, where Rubisco is the main carboxylase in phase IV. Indeed, results of *K. blossfeldiana* 'Saja' confirmed that Rubisco was the main carboxylase. Additionally, carbohydrate analysis showed that starch accumulated in *Phalaenopsis* during phase IV which indicated that Rubisco also active as a carboxylase, alongside PEPC. This chapter discusses that having both carboxylases simultaneously active may lead to double carboxylation and futile cycling of CO_2, but that it might also serve as a mechanism for photoprotection.

Chapter 6 summarizes and reflects upon the concepts and conclusions of the previous chapters. This discussion focusses on how processes that are determined within diel cycles link to plant growth, which is determined over a longer period of time. The methodology that was used for gas exchange measurements is reflected upon. In this chapter, the effect of other environmental factors such as red: far-red and CO_2 on *Phalaenopsis* cultivation are examined. This chapter furthermore described perspectives of the results of this thesis for commercial *Phalaenopsis* cultivation. Linking together the results of different chapters, it is demonstrated that diel CO_2 uptake measured on a leaf can be used as a proxy for flowering quality, and could thus function as an early selection criterium when phenotyping in *Phalaenopsis* breeding. Additionally, some suggestions are given for future research, building on knowledge produced in this thesis, e.g. how to improve the conceptual framework. This chapter is concluded with some personal remarks on the study of CAM and photosynthesis research.

Samenvatting

Crassulacean Acid Metabolism (CAM) is een aanpassing in het fotosynthesesysteem van planten, die is ontstaan als gevolg van droogtestress, al dan niet in combinatie met hoge temperaturen. CAM-planten komen niet alleen voor in gebieden met een droog klimaat, ze groeien ook in tropische regenwouden, bijvoorbeeld als epifyten, die geen directe toegang hebben tot een rijke voedingsbodem. CAM-planten hebben erg weinig water nodig. Zij nemen 's nachts CO_2 op, waardoor ze op het heetst van de dag hun huidmondjes kunnen dichthouden. CAM wordt over het algemeen niet gezien als een eigenschap die erg nuttig is in landbouw, want CAM-planten worden (vaak ten onrechte) bestempeld als weinig productief. Daarom is er relatief weinig onderzoek gedaan naar CAM-planten, in vergelijking met C_3 en C_4 planten. Dit proefschrift heeft als doel om de kennis van CAM te vergroten, door onderzoek te doen naar de economisch belangrijke potorchidee *Phalaenopsis* bij verschillende combinaties van temperatuur en licht. Het effect van deze behandelingen is bestudeerd op verschillende biologische integratieniveaus en op verschillende tijdsschalen, variërend van blad- tot plantniveau, en van secondes tot en met de gehele teelt.

In hoofdstuk 1 wordt het begrip CAM geïntroduceerd. Dit hoofdstuk beschrijft hoe de 24-uurs cyclus van CO_2-opname en CO_2-vastlegging kan worden onderverdeeld in vier kenmerkende fases. Ook worden in dit hoofdstuk de energetische meerkosten van CAM toegelicht. Daarnaast beschrijft dit hoofdstuk de grote fenotypische variatie waarop CAM zich kan uiten. CAM-planten worden vaak over het hoofd gezien in de overwegingen naar een toekomstbestendige landbouw, al wordt er soms gesproken over het zodanig veranderen van C_3 planten dat ook zij in staat zijn CAM-fotosynthese te gebruiken. De tweede helft van hoofdstuk 1 spitst zich toe op de teelt van CAM-orchidee *Phalaenopsis*, en worden de effecten van temperatuur, licht en CO_2 op groei en ontwikkeling van *Phalaenopsis* samengevat.

Hoofdstuk 2 introduceert een conceptueel denkkader om de groei van CAM-planten te beschrijven. Na literatuuronderzoek bleek dat er geen mechanistische simulatiemodellen voor CAM plantgroei bestaan. Het denkkader bestaat uit drie modules die processen beschrijven gerelateerd aan 1) CAM fotosynthese; 2) de verdeling van assimilaten over verschillende vormen van koolstofopslag; 3) de verdeling van assimilaten over verschillende plantorganen. Het denkkader beschrijft CAM-fysiologie op verschillende biologische integratieniveaus en tijdsschalen, en kan fungeren als basis voor de ontwikkeling van een

mechanistisch gewasgroeimodel van CAM-planten. Op basis van dit denkkader konden een aantal kennishiaten rondom CAM-fysiologie vastgesteld worden. Zo bleek dat, er nog veel onduidelijk is over hoe assimilaten worden verwerkt en verdeeld binnen een CAM-plant. Ook worden mogelijke gevolgen van het bestuderen van CAM in een niet-gelimiteerde omgeving onderzocht. Het denkkader diende als basis voor de volgende hoofdstukken in dit proefschrift.

Hoofdstuk 3 beschrijft de variatie tussen verschillende *Phalaenopsis*-genotypen. In twee experimenten met 19 en 14 genotypen zijn de effecten van temperatuur en licht op de vegetatieve groeikenmerken bestudeerd. Ook zijn de effecten van deze behandelingen onderzocht tijdens de (uit)bloei van de planten. Hiërarchische componentanalyse gaf inzicht in hoe verschillende plantkenmerken met elkaar verband houden, en hoe deze bijdragen aan groei en ontwikkeling van *Phalaenopsis*. Zo hadden planten die zijn opgekweekt bij 31°C veel minder plant- en wortelgewicht in vergelijking met planten die zijn opgekweekt bij 27°C, al was daar de bladafsplitsingssnelheid hoger. Een toename in lichtintensiteit (van 60 µmol m^{-2} s^{-1} naar 140 µmol m^{-2} s^{-1}) leidde tot een versnelling van groei (gewichtstoename) en ontwikkeling (bladafsplitsing). Een toename van licht en temperatuur in de vegetatieve fase leidde tot een toename van het aantal bloemtakken, en van het aantal bloemknoppen en open bloemen. In dit hoofdstuk wordt aangetoond dat de vegetatieve fase een goede indicatie kan geven van latere bloeikwaliteit, ondanks aanzienlijke genotypische en fenotypische variatie. Vegetatieve plantkenmerken zoals het aantal bladeren en het plantgewicht zijn indicatief voor het aantal bloemtakken en bloemtakgewicht.

In hoofdstuk 4 worden de fysiologische verschillen onderzocht die ten grondslag liggen aan de groei en ontwikkeling van *Phalaenopsis*. Vegetatieve planten van twee genotypen zijn opgekweekt bij dezelfde temperatuur- en lichtbehandelingen als gebruikt in hoofdstuk 3. Metingen van het verloop van de CO_2 opname en koolstofassimilatie gedurende 24 uur werden gecombineerd met langetermijn-metingen aan groei en ontwikkeling. Per genotype is een hoofdcomponentenanalyse (PCA) uitgevoerd om te bepalen welke van de gemeten kenmerken het meest verklarend waren voor de verschillen tussen de genotypen. Daaruit bleek dat de vijf processen die het meest varieerden verdeeld waren over de drie modules van het conceptuele denkkader (hoofdstuk 2). De genotypen verschilden in de duur van CAM-fasen, transpiratie en CO_2-opname. Daarnaast waren er verschillen zichtbaar in de hoeveelheid zetmeel die gedurende de dag werd opgebouwd en gedurende de nacht werd afgebroken. Ook traden er verschillen op in wortelgewicht, het aantal bladeren en de dikte van de bladeren. Er bleek

duidelijk een relatie te bestaan tussen CO_2-opname gemeten over 24 uur, en het vegetatieve drooggewicht van een *Phalaenopsis*plant. Echter, deze relatie geldt alleen als de CO_2-opname wordt uitgedrukt per plant, en niet wanneer deze wordt uitgedrukt per m^2 bladoppervlak.

CAM-fase IV vindt plaats op het einde van de dag en wordt beschouwd als een belangrijke fase, omdat hierin een substantiële hoeveelheid CO_2 opgenomen kan worden die direct bijdraagt aan de productiviteit van CAM-planten. Daarom is in hoofdstuk 5 onderzocht in welke mate C_3 (via Rubisco) of C_4 (via PEPC) carboxylatie bijdragen aan CO_2-opname in twee CAM planten, te weten *Phalaenopsis* 'Sacramento' en *Kalanchoe blossfeldiana* 'Saja'. Metingen van gasuitwisseling en chlorofylfluorescentie zijn uitgevoerd bij 21 en 2% zuurstof. Daarnaast zijn deze metingen uitgevoerd terwijl gedurende korte periodes het licht uitgeschakeld. Hieruit bleek dat PEPC de belangrijkste carboxylase is om CO_2 vast te leggen gedurende fase IV in *Phalaenopsis* 'Sacramento'. Dit komt niet overeen met eerder gevonden resultaten aan andere CAM-planten, waarvan steeds werd gezegd dat Rubsico de belangrijkste carboxylase is in CAM-fase IV. Dit bleek ook uit de metingen aan *Kalanchoe blossfeldiana* 'Saja', waar Rubisco inderdaad de belangrijkste carboxylase was. In fase IV bleek dat er sprake was van zetmeelaccumulatie in *Phalaenopsis* 'Sacramento' . Dit geeft aan dat, naast PEPC, Rubisco tegelijkertijd ook actief was. In dit hoofdstuk worden de mogelijke consequenties hiervan besproken. Het gelijktijdig actief zijn van beide carboxylases zou kunnen leiden tot dubbele carboxylatie, en dus tot energieverspilling door het onnodig vastleggen en vrijmaken van CO_2, maar het zou ook kunnen dienen als fotoprotectiemechanisme.

In hoofdstuk 6 worden de resultaten en conclusies uit de voorgaande hoofdstukken samengenomen en beschouwd. In deze beschouwing ligt de nadruk op de koppeling van processen die zich steeds in een 24 uur-cyclus afspelen met processen die plaatsvinden over een langere tijdsperiode, zoals groei en ontwikkeling. Daarnaast wordt de methode die gebruikt is om gasuitwisseling te meten onder de loep genomen, en wordt het effect van andere omgevingsfactoren, zoals verschillende verhoudingen van rood en verrood licht, op de opkweek van *Phalaenopsis* besproken. Doordat de resultaten van verschillende hoofdstukken aan elkaar gekoppeld worden, blijkt in dit hoofdstuk dat de CO_2 opname gemeten over 24 uur gebruikt kan worden als indicatie voor bloeikwaliteit van de plant. Dit zou kunnen dienen als een selectiecriterium dat in een vroeg stadium kan worden ingezet voor fenotypering. In dit hoofdstuk wordt tevens besproken hoe de resultaten die gevonden zijn in dit proefschrift ingezet zouden kunnen worden in de teelt van *Phalaenopsis*. Ook wordt een aantal suggesties

gedaan over hoe toekomstig onderzoek zou kunnen voortbouwen op dit proefschrift, bijvoorbeeld door verbetering van het conceptuele denkkader. Dit hoofdstuk sluit af met een aantal persoonlijke opmerkingen over onderzoek naar fotosynthese in het algemeen en CAM in het bijzonder.

Korte samenvatting

Fotosynthese is het basisproces in planten, waarin CO_2 wordt opgenomen via de huidmondjes, en vervolgens wordt vastgelegd in suikers voor groei. Crassulacean Acid Metabolism (CAM) is een aanpassing in het fotosynthesesysteem, ontstaan door blootstelling aan droogte en hoge temperaturen. CAM-planten komen overal voor, maar er is nog niet veel onderzoek gedaan naar CAM-planten. Daarom heb ik onderzoek gedaan naar de pot-orchidee *Phalaenopsis,* bij verschillende combinaties van temperatuur en licht.

Allereerst heb ik een schematisch model gemaakt. Daarin worden processen die belangrijk zijn voor groei en ontwikkeling van CAM-planten aan elkaar gekoppeld. Het model bestaat uit drie delen: 1) fotosynthese (het opnemen van CO_2); 2) de verdeling van suikers binnenin de plant; 3) groei van planten.

Uit experimenten (hoofdstuk 3) met *Phalaenopsis* bleek het volgende: 1) een hoge temperatuur leidt tot kleinere planten met minder wortels; 2) meer licht leidt tot grotere planten met meer bladeren. 3) de combinatie van hogere temperatuur met meer licht tijdens de vegetatieve fase (als de planten nog niet bloeien) leidt daarna tot meer bloemtakken en meer bloemen.

Vervolgens heb ik in meer detail onderzocht hoe dat komt (hoofdstuk 4). Ik heb allerlei soorten metingen gecombineerd, zoals CO_2-opname, hoeveelheid suikers in het blad, en groei. Dit onderzoek liet ook zien dat meting van CO_2-opname gebruikt kan worden om de groeisnelheid van een plant te voorspellen. Daardoor kun je eerder iets zeggen over hoe snel een plant groeit, zonder dat je de opkweektijd hoeft af te wachten.

De manier waarop CO_2 wordt vastgelegd door de plant verandert over 24 uur. Het was nog niet helemaal duidelijk hoe dat bij *Phalaenopsis* gebeurde, vooral niet aan het einde van de dag. Uit mijn onderzoek (hoofdstuk 5) bleek dat *Phalaenopsis* dat anders doet dan de meeste CAM-planten. De *Phalaenopsis*-manier lijkt minder efficiënt te zijn, maar het zou ook kunnen dat de plant daardoor beter beschermd is tegen teveel licht.

In hoofdstuk 6 kijk ik terug en koppel ik alle resultaten aan elkaar. Ik laat zien dat metingen aan CO_2 opname gebruikt kunnen worden om de kwaliteit van bloeiende planten te voorspellen en zo snel planten met een hoge kwaliteit te selecteren. Ook doe ik een aantal suggesties voor het gebruik van mijn resultaten in de praktijk, en voor toekomstig onderzoek.

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About the author

Evelien van Tongerlo was born on August 5th, 1987 in 's Hertogenbosch, The Netherlands. Being the daughter of cut flower growers, she spent most of her childhood playing in and around a greenhouse. After finishing high school (Havo, because she didn't feel the need to go to university anyway), she moved to Nijmegen for her BSc in Biology at the HAN university of applied sciences in order to become a high school teacher. After graduation she realised that teaching didn't make her happy, and started to broaden her horizon in order to find something that was more to her liking. She studied Christian theology for a bit, and worked as a car mechanic on both Citroën and Porsche classic cars. Despite her love for cars, university kept calling her until she could no longer resist her thirst for knowledge. So off she went, for a MSc in Plant Sciences with a specialization in, what else, greenhouse horticulture at Wageningen University. For her MSc thesis project, Evelien worked on the development of a dynamic functional-structural plant model (FSPM) for tomato under the supervision of Ep Heuvelink and Pieter de Visser. During her MSc she intentionally chose courses that focused on the practical side of science, attempting to stay away from fundamental research because she was sure she wouldn't stay in academia anyway. But she was wrong again. She conducted her internship at Anthura in Bleiswijk, where she conducted measurements in to the small hours of the night. It was right then and there, in the midst of the greenhouse, surrounded by thousands of plants, that she decided to pursue the PhD that she got offered, which allowed her to continue to work with Phalaenopsis. The results of her research are presented in this thesis.

During her PhD, Evelien was involved in the promotion of photosynthesis research at a university-wide level, and she is co-founder of the study group 'Sharing Knowledge in Photosynthesis Research' (SKiPR), which aims to share knowledge on all things photosynthesis, stimulate collaborations within, and outside of Wageningen University. She was also involved

in the organisation of an international mini-symposium: 'Photosynthesis: making it better and its value as tool for addressing societal needs', which had Donald Ort as the main speaker.

Currently, Evelien is living in Nijmegen with her partner Evelien (this is not a typo) and their ridiculously fluffy cat Charlie. Evelien (the one that wrote this thesis, not the other one), is currently working as freelance data analyst and cybersecurity consultant.

List of publications

Publications in peer-reviewed journals

Davis, S. C., Simpson, J., Gil Vega, K. del C., Niechayev, N. A., **van Tongerlo, E.**, Castano, N. H., … Búrquez, A. (2019). Undervalued potential of crassulacean acid metabolism for current and future agricultural production. Journal of Experimental Botany, 70(22), 6521–6537.

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Other publications

van Tongerlo, E., Marcelis, L.F.M., & van Velden, P. (2019). Snellere aanleg en uitgroei van bloemtakken met verrood licht. Onder Glas, 16, 14-15. (In Dutch)

van Tongerlo, E., Marcelis, L.F.M., & van Velden, P. (2020). Flower stems form and develop faster with far-red light. In Greenhouses, 3, 46-47.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities).

Review of literature (4.5 ECTS)

- Conceptual framework for crops utilizing CAM: scaling up from diel photosynthesis at leaf level to crop growth

Post-graduate courses (5.4 ECTS)

- Plant Environmental Physiology Group (PEPG) techniques workshop; Plant Environmental Physiology Group, SEB (2016)
- Masterclass Technovit; Laboratory of Cell biology / Wageningen Light Microscopy Centre (2016)
- Introduction to R for statistical analysis; PE&RC (2016)
- 11th International metabolomics workshop; Leiden University (2019)
- Data visualization with David McCandless; YoungWUR (2020)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics; PE&RC (2016)

Laboratory training and working visits (1.5 ECTS)

- 11th International metabolomics hands-on workshop; Leiden University (2019)

Invited review of journal manuscripts (2 ECTS)

- Frontiers in Plant Science: developmental control of CAM expression during early seedling growth of Phalaenopsis orchid (2018)
- Journal of Experimental Botany: starch degradation pathways in plants with Crassulacean acid metabolism (2020)

Competence strengthening / skills courses (1.9 ECTS)

- Competence Assessment (COA); WGS (2016)
- Teknowlogy workshops: how to TEDx / effective networking; NWO (2017)
- Effective behaviour; WGS (2018)
- Last stretch of the PhD programme; WGS (2020)

- Writing propositions for your PhD; WGS (2020)

Scientific integrity / ethics in science activity (0.6 ECTS)

- Scientific integrity; WGS (2019)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- PE&RC First years weekend (2016)
- PE&RC Day (2016)

National scientific meetings / local seminars / discussion groups (9.9 ECTS)

- FLOP discussion group (2016-2020)
- Annual meeting experimental plant sciences (2017)
- Working conference photosynthesis (2018)
- Light and photosynthesis study group (2020-2021)
- SKiPR: sharing knowledge in photosynthesis research (2019-2020)

International symposia, workshops and conferences (9.5 ECTS)

- Plant control by LED Light; Wageningen (2017)
- Biology of CAM plants; AZ, USA (2018)
- Gordon research conference: CO2 assimilation in plants from genome to biome; ME, USA (2019)
- Vertifarm; Wageningen (2019)
- Mini-symposium Photosynthesis; Wageningen (2020)

Societally relevant exposure (0.2 ECTS)

- Article: onder glas (2019)

Lecturing / supervision of practicals / tutorials (5.7 ECTS)

- Advanced methods in plant-climate research (2016-2018)
- Physiology and development of plants in horticulture (2017-2019)
- Concepts in environmental plant physiology (2017-2018)
- Research methodology in plant sciences (2017-2018)

MSc thesis supervision (9 ECTS)

- Light and temperature effect on diurnal photosynthetic metabolites of Phalaenopsis
- The effect of far-red lighting on photosynthesis and crop development in Phalaenopsis
- Photosynthetic difference among genotypes and the effect of red and far-red light on diurnal photosynthesis of Phalaenopsis in reproductive stage
- Effects of sink limitation in photosynthesis of young vegetative Phalaenopsis

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