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Role of primary metabolites and pigments during floral morphogenesis in *Coccinia grandis* (L).Viogt (Cucurbitaceae)

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Abstract

Flowering is one of the energy consuming events in a lifecycle of plants and is controlled by various exogenous and endogenous factors. Carbohydrates produced from photosynthesis are the primary energy source that is mobilized to sink tissues during flower development. *Coccinia grandis* (ivy gourd), a dioecious cucurbit exhibits different rates of development during floral morphogenesis. Present investigation estimated the carbohydrate and protein supply during floral morphogenesis and revealed their role as energy source in both male and female flower development. However, the chlorophyll content was found decreased both in male and female plants during flower development progresses. The changes in flower colour from green to white during development may be due to the loss of pigments *viz*, chlorophyll, anthocyanins and carotenoids and, this may be an adaptive mechanism towards pollinators for easy identification from the greenery background.

Keywords: carbohydrate, chlorophyll, protein, floral morphogenesis.

Introduction

Flowering is an essential physiological process in the life cycle of angiosperms, being the central process of species reproduction. The formation of flower buds is also a fundamental factor in productivity. Differentiation of flower is a highly regulated process, as the development progress the changes will happen in the colour and shape. Flowers are complex structures, developed from florally determined meristem which in turn proliferated to form various floral organs like sepals, petals, stamens and carpels. The flowering transition is controlled by inheritance as well as internal and external factors accompanied by various changes in the morphology, biochemistry, physiology, and cytology of the buds leading to the formation of reproductive structures (Chen *et al.,* 2009).

The carbohydrate metabolism is the major source of energy for the regulation of developmental processes from embryo to senescence of flower (Gibson 2005; Hanson & Smeekens 2009; Rolland *et al.,* 2006). The two main carbohydrates involved in flower nutrition are sucrose, as the main transportable form (Winter & Huber 2000) and, the starch, which is actively metabolized during the development of floral tissues (Rodrigo *et al.,* 2000). Like leaves, the reproductive structures of many plants are also photosynthetically active and, thus can fix substantial amount of carbon. Photosynthesis has been recorded in almost

all sterile and fertile parts of the flower such as bracts, sepals, petals, anthers, carpel and also in fruits (Aschan & Pfanz 2003; Aschan *et al.,* 2005). Estimations of the contribution of reproductive photosynthetic components to their own carbon requirement vary depend on the species, organ, developmental phase, and ambient conditions (Birkhold *et al.,* 1992).

The candidate species *Coccinia grandis* (ivy gourd) is dioecious belonging to Cucurbitaceae. The species is unique by the presence of heteromorphic sex chromosomes (Roy 1971). Due to its dioecious nature, plant exhibits different rates of development during floral morphogenesis. The male and female flowers showed structural similarity in their outer whorls; both possessed five sepals and five united petals. However, the sexual whorls were quite variable and have three fused stamens in the male and an inferior ovary with a single style and a trifid stigma in the female flower. There is a necessity to understand the mechanism of the fundamental changes in the physiology of flowers during development, especially in the dioecious plants because they differ only in their flowering processes, most of which are genetically controlled. However, studies based on the physiological changes involved during flowering process are limited. The present study analyzed the significance of primary metabolites such as carbohydrates and proteins and pigment profile during the different stages of flower development in the male and female plants of *C. grandis*.

Materials and Methods

Plant material

The male and female plants of the *C.grandis* were grown in a greenhouse controlled temperature (27- 30^0C) and humidity (60-70%) at the Department of Botany, University of Kerala. The flowers and floral buds collected from male and female plants were used for the study.

1. Floral morphology and development

Morphology of male and female flowers, floral buds, and floral parts of *C. grandis* was studied in detail with the help of macroscopic and microscopic techniques. Fresh floral samples of *C.grandis* were examined under a stereomicroscope, connected to an ultra-scope digital camera (Stereomicroscope System SZ61, Olympus) to study the micro-morphological characters. Measurements were taken with SwiftCam Imaging II and analyzed statistically (SPSS, version 20).

2. Physiological studies

The fresh samples from male and female flowers of *C.grandis*at different developmental stages (stage I-VI) (figure 1, 2), were harvested immediately and transferred to the liquid nitrogen. The primary energy resources (carbohydrates, protein) and major pigments (chlorophyll, carotenoids, and anthocyanins) were quantified from each sample and all the data were statistically analyzed with SPSS (Version 17).

2.1. Carbohydrate estimation

The quantification of carbohydrates was done according to the Anthrone method with glucose as standard (Sigma-Aldrich) (Loewus 1952). One gram of sample was hydrolyzed with 5 ml of 2.5N HCl for 3 hours and neutralized with Na_2CO_3 until the formation of effervescence; centrifuged the mixture and collected the supernatant. Then 4ml of anthrone reagent (200mg anthrone powder in 100ml ice cold 95% H_2SO_4) was added to the solution. The reaction mixture was heated for 8 minutes, and then cooled rapidly.The absorbance at 625nm was scored in a spectrophotometer (UV-PharmaSpec-1700, Shimadzu).

2.2. Protein estimation

Protein quantification was conducted according to Bradford assay using BSA (Sigma-Aldrich) as standard (Bradford 1976). The 100 mg of each sample were homogenizing with 5 ml phosphate-buffered saline (PBS). Added 5ml of diluted dye binding solution (Bradford reagent) to each tube mixed well and allowed for to develop blue colour from the red dye for at least 5 minutes. Absorbance at 595nm was measured in a spectrophotometer (UV-PharmaSpec-1700, Shimadzu).

2.3. Quantification of pigments

The quantification of pigments, total chlorophyll, chlorophyll a, and b, carotenoids, and anthocyanins were carried out according to Arnon (1965). For quantification, 100 mg of fresh sample was extracted with 80% acetone (Merck) and centrifuged at 7000 rpm for 20 minutes (Centrifuge 5430R, Eppendorf). The supernatant was collected, and absorbance at 663,646, 470, and 537 nm were noted for chlorophyll a, chlorophyll b, carotenoid, and anthocyanin respectively, using a spectrophotometer (UV-PharmaSpec-1700, Shimadzu).

Results and Discussion

The flowers have evolved unique mechanisms to attract pollinators for increasing the rate of

reproductive success, while the exact mechanism behind this is still in debate (Cooley *et al*. 2008). Development of a flower is affected by different factors, including genetic, temperature, light, and seasonal variations (Oster *et al*. 2002; Erwin 2007). Most of the plants adjust the timing of crucial developmental processes that occur timely in each season which helps to maximize their chances of survival and reproductive success (Hastings & Follett 2001; Yanovsky *et al*., 2003). *C. grandis* is a fast-growing vine which makes flowers, and fruits throughout the year, while the number and frequency of flower were varied. Both the male and female flowers are produced from the axiliary meristems; in male plants, more than one floral bud was developed from a single axis, while in females, only a single flower was raised, which developed into the fruit.

The male and female plants of *C. grandis* are distinguished only by their reproductive whorls. Both male and female flowers of *C. grandis* have five green sepals and five white petals each. The male flowers had a trilobite anther with numerous pollen grains, while the female flowers consisted of a trilobite stigma and an elongated inferior ovary. However, the development of flowers in male and female plants showed variations in *C. grandis*. In the present study, the whole developmental process in male and female flowers of *C. grandis* was broadly categorized into six stages based on macro and micro-morphological observations (figure 1). They includes sepal and petal development (stage I), formation of anther (male) and stigma (female) (stage II), development of pollen grain and ovule (stage III), mature bud at one day before flowering (stage IV), flower (stage V), and senescence flower (stage VI).

The sepal and petal development (stage I) was identical in both male and female candidates, five whorls of sepals are raised from the floral meristem (figure 2a), and then the petals are formed inner to the sepals in a sequential manner (figure 2 b, g). The petals are green coloured at the initial stages of their development, which turned into white during the progress of development. The developmental events of stage II was quite different in both sexes, includes formation of anther in male and stigma in the female (figure 2 c, h). After the formation of anther meristem pollen grains were developed (stage III), the mature anther of *C. grandis* had numerous pollen grains in their folding with a thick filament (figure e, f); while in females, the stage III included the formation of an inferior ovary and ovule (figure l, k). A notable colour difference was observed during the development of anther, it was green in the beginning followed by pale yellow on maturation (figure 2 e, f), while there was no remarkable colour change in female. The stage IV of flower development in *C. grandis* is considered as mature bud before the flowering day, where all floral whorls except ovary (sepals, petals, anther, and stigma) have attained their maximum growth. The flowering day in male and female was included as the stage V followed by senescence (next day of flowering) stage VI, in this stage floral sepals, petals, anther and stigma were wilted, except ovary in the female flower, which turned into fruit after pollination. During the entire process of development, remarkable changes were noticed in the colour, size and shape of each floral whorl, especially for petals. The petals of mature flower were white, which makes petals showy and can easily be identified by pollinators from the green background of leaves. This may be an adaptive mechanism of the flower for attracting their pollinators.

Flowering is the most energy-consuming stage of the annual cycle of development in plants (Finazzo *et al*., 1994). The flower development consumes more energy than they assimilate; they constitute the most energetic sink organ during the reproductive period. The carbohydrates are one of the energy sources needed for regular metabolic activities in plants; they are also important in the growth, development, and reproduction. The level of carbohydrates have been postulated as one of the key determining factors in initiation of inflorescence as well as development of floral organs (Rodrigo *et al.,* 2000), and in the formation, development, and maturation of fruits (Ruiz *et al*., 2001; Iglesias *et al*., 2003). A complex carbohydrate flux is the main source-sink of interactions in the reproductive development (Lebon *et al*., 2008). The carbohydrate metabolism rate in both the male and female floral developmental stages in *C. grandis* showed variations (figure 3 a). In males, concentration of carbohydrates was almost stable in all the stages of development, while a sudden decline was noticed during the senescence stage (stage VI). The utilization of carbohydrates in females differed from that of male, and observed a gradual decline during the progress of flowering and senescence. The soluble carbohydrates showed an increase in the floral development, *i.e.*, from bud to fully open bloom, after which it was found declining towards senescence. Carbohydrate supply from bud to flower in *C. grandis* has pointed out the importance of carbon nutrients as a primary energy source during flower development in male and female *C. grandis*. According to Hieke *et al*., (2002), the

carbon nutrient required for flower and fruit development is assumed to originate either from photosynthesis in the leaves or from branches or root reserves.

The study noticed an increase in soluble proteins up to bud break, then a decrease in the flowering stage, and a subsequent increase as the senescence progressed in the male flowers. However, in the case of female flowers, the protein concentration increased up to flowering and then showed a slight decline during the senescence (figure 3b). The decline in total soluble protein after full flower opening can be correlated with the loss of proteins, as suggested by Kenis *et al*., (1985) and Woodson & Handa (1987). Senescence is the final phase in the ontogeny of an organ or the whole plant involving a series of irreversible events that lead to endogenously controlled cellular breakdown and death (Leopold 1975). Protein degradation is an important feature of dismantling of membranes (Woolhouse 1984). The progressive destabilization of membrane bilayer accompanying senescence may likely be due to loss of membrane protein function (Thompson 1974).

The changes in shape and colour of flowers are tightly controlled by multiple genes. During this process, each part of the flower shows a distinct species-specific pattern of colour change. The various pigments responsible for the different colours in the flowers include chlorophyll (green), anthocyanins (red/purple and blue/ black), carotenoids (orange/yellow), and betalains (yellow/red). The change in colour during floral morphogenesis was accomplished either by producing or blocking specific pigments that they wanted. The floral morphogenesis in *C. grandis* was accomplished by a change in the green coloured floral bud to green sepals, colourless petals (white), pale yellowish/ greenish anther in male and stigma in female, and, green coloured ovary, which later developed into green fruit with white stripes followed by orange or red during the ripening stage. The quantity of pigments such as total chlorophyll, chlorophyll a, and b, carotenoids, and anthocyanins estimated during floral morphogenesis of *C. grandis* is shown (figure 4, 5).

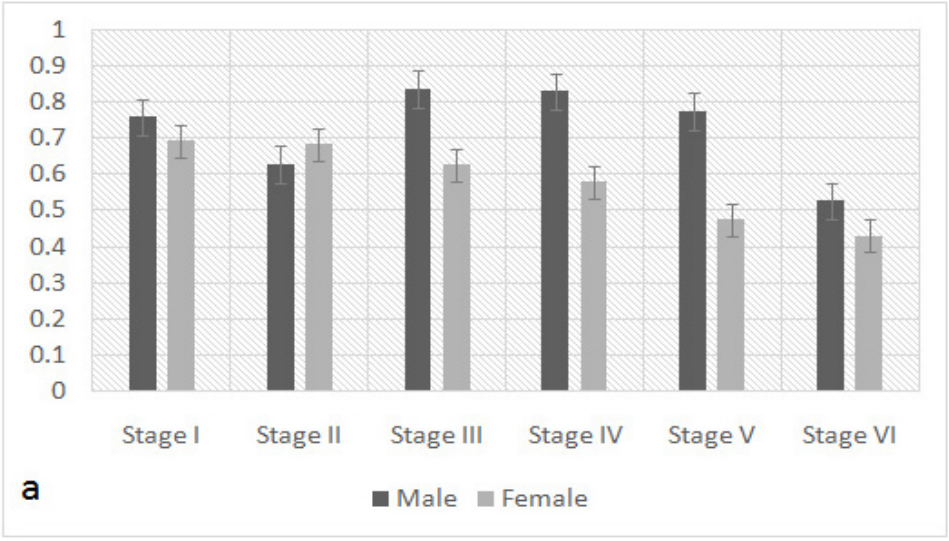
The chlorophyll is a group of green coloured pigments that are responsible for photosynthesis in plants. Total chlorophyll concentrations of both male and female flowers decreased during development from stage I to VI (figure 4a). In both plants, the decrease was gradual except for stage IV (one day before flowering) and V (flowering day) of female flowers. Chlorophyll a and b also declined in all stages during the flower development. The chlorophyll a/b ratio observed in reproductive organs, was an indicator of chlorophyll concentrations and photosynthetic rates (Blanke & Lenz 1989). Chlorophyll a/b ratio fluctuated in both male and female flowers of *C. grandis* over the studied period are a reliable indicator of net photosynthesis occurrence. The decreased nature of chlorophyll content during the progress of flowering and senescence in both male and female flowers revealed an intense degradation of chlorophyll in the floral organs of *C. grandis* during the development. The loss of chlorophylls during flower development in *C. grandis* is a crucial trait that enables flowers to be visually distinguished against a background of leaves when the flowers are ready to offer rewards to pollinators. According to Tanaka *et al*. (2008) and Sakuta & Ohmiya (2011), the loss of green pigments in the flower petals and accumulation of other pigments like anthocyanin and carotenoids are common traits exhibited by flowering plants.

The two essential pigments, carotenoids and anthocyanins analyzed during the investigation revealed the presence of high quantity of the pigments in the floral buds of male and female plants, while the pigments started declining as the flowering progresses (figure 5). The decrease in the quantity of pigments during floral morphogenesis may be the cause for the white coloured petals in *C. grandis* flowers. Initially, the floral buds have anthocyanins and carotenoids, and the loss of pigments during later stages may lead to the transition of flower colour from green to white. In general, acyanic flower colours like white or ivory are caused by blocking the anthocyanin pathway or due to expression of recessive alleles.

The present study revealed the significance of carbohydrate and protein metabolism during flower development in male and female plants of *C. grandis*. Primary metabolites including carbohydrate and protein supply from bud to flower development in *C. grandis* confirmed the importance of carbon nutrients as energy source in male and female flowers. The study correlates the change in flower colour with that of pigment loss during floral development and suggests that, it may be an adaptive mechanism of these flowers towards pollinators for easy identification from the green background created by leaves.

Figure 1: Different developmental stages of male and female flowers selected for physiological and biochemical studies

Figure 2: Morphological variations in the progress of floral morphogenesis: a-sepal developed from floral meristem, b-petal development in male, c- anther meristem formation in male, d, e-maturation stages of anther-f- dehisced anther, g-petal development in female, h, j-stages of stigma development, l,k-inferior ovary formation.



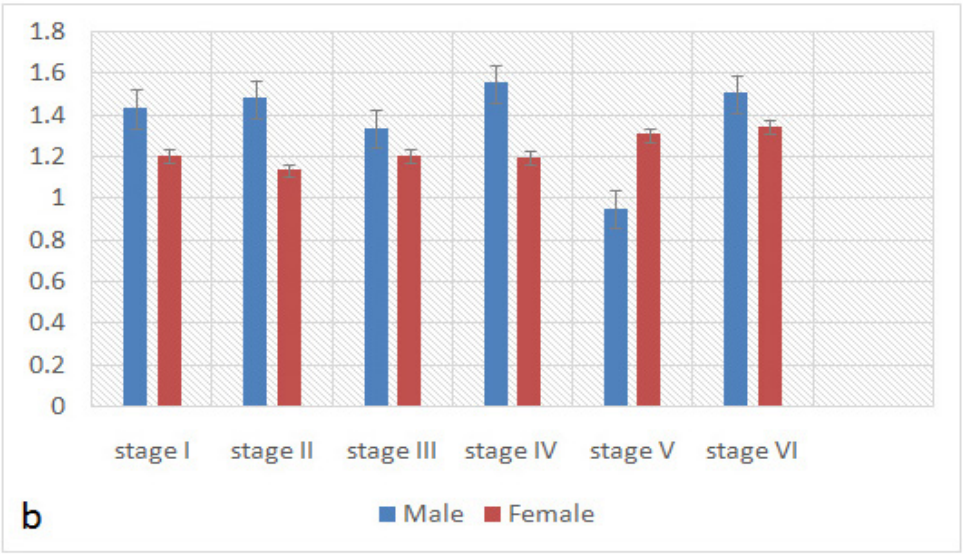


Figure 3: Quantification of protein and carbohydrates during floral development in *C. grandis*:
a- total carbohydrate and b- protein

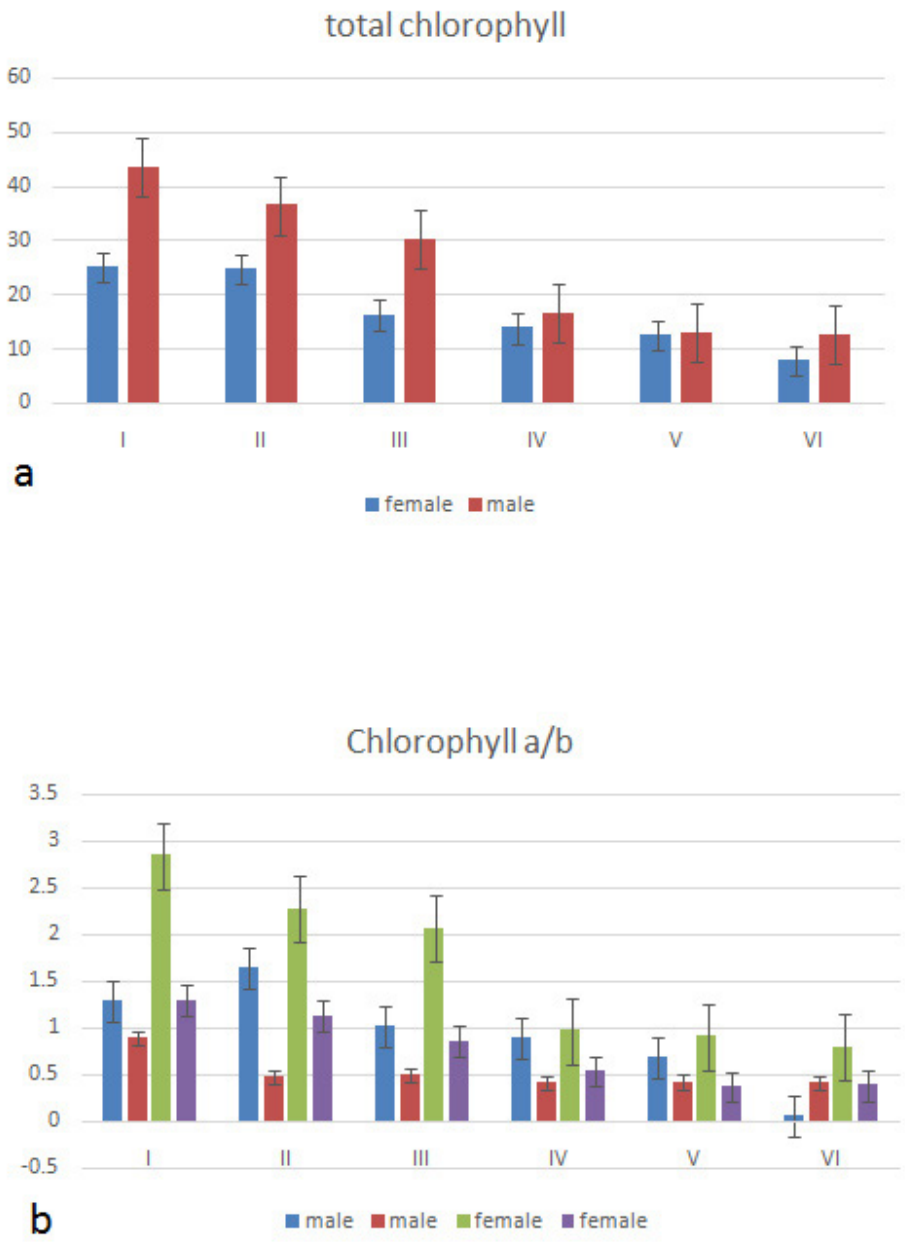
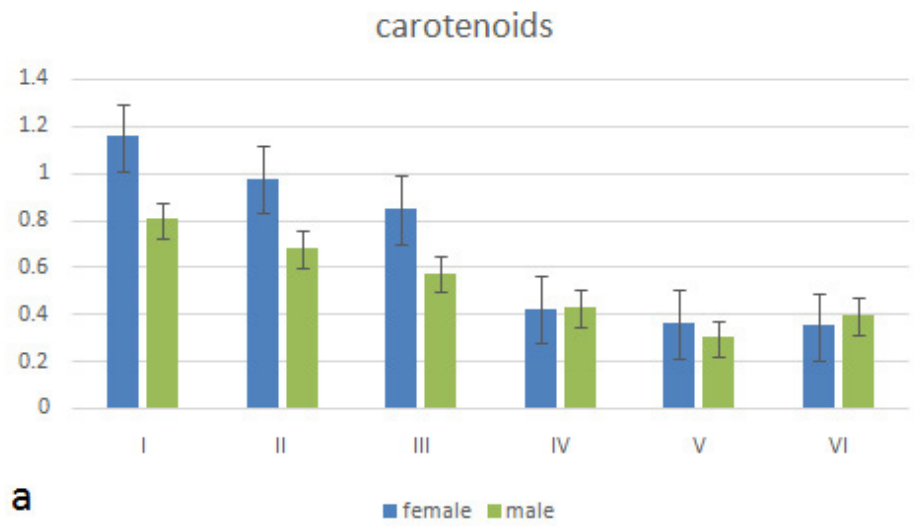


Figure 4: Quantification of chlorophylls during floral morphogenesis: a-total chlorophyll and b-chlorophyll a (blue and green), chlorophyll b (brown and violet)



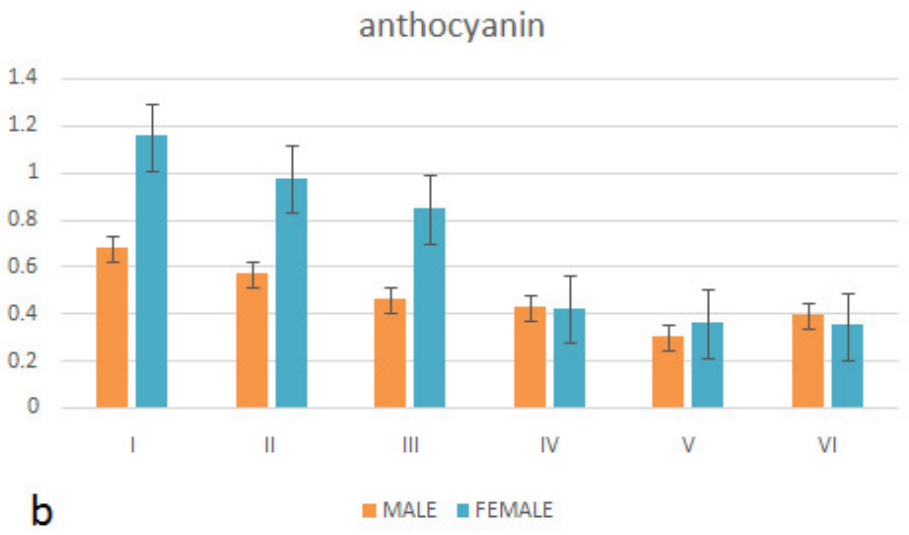


Figure 5: Quantification of pigments during floral development: a- total carotenoids and b-anthocyanins

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