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Original article

Role of carbohydrates in petal blackening and lack of flower opening in cut lotus (*Nelumbo nucifera*) flowers

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ABSTRACT

Lotus flowers (*Nelumbo nucifera* Gaertn.) are sold as stems with terminal buds that are about to open, to be used in Buddhist religious offerings. The buds fail to open if the cut stems are placed in water. Moreover, the petals rapidly turn black. This study investigated whether this might be due to a lack of carbohydrates. The inclusion of different sugars in the vase water, together with an adequate antimicrobial compound, had no effect on petal blackening and did not promote flower opening. By contrast, cutting the buds at a slightly more mature stage of development resulted in full flower opening. However, the levels of glucose, fructose, or sucrose in the white petals were the same at the later date of harvest as at the earlier date; thus, this did not explain the effect of cutting at an advanced stage of development. It was concluded that a lack of sugars does not seem to explain petal blackening or a lack of flower opening in lotus flowers that are cut at the normal harvest stage (bud stage).

Introduction

Lotus flowers (*Nelumbo nucifera* Gaertn.) are commercially sold as closed buds. The stems are brought to a Buddhist temple as an offering. If the stems are placed in water, the buds do not open, and the outer petals rapidly turn black. Treatment with 1-methylcyclopropene (1-MCP), an ethylene antagonist, delayed petal blackening and this indicated that blackening is regulated by endogenous ethylene (Imsabai et al., 2010). Blackening was delayed after treatment with hormones such as gibberellic acid and cytokinins (Imsabai and van Doorn, 2013), and was delayed by decreasing the rate of transpiration (Imsabai et al., 2013). These data suggested that the early blackening was partially due to adverse water relations, and by a low level of hormones such as gibberellic acid and cytokinins. However, none of these treatments caused bud opening.

After harvest, the cut lotus stalk with its terminal floral bud has no leaves, thus is not in contact with a potential source of carbohydrates. Since the stems are placed at relatively low light levels (about 15 μmol/m^2/s), net photosynthesis is likely absent. This means that petal sugar levels might decrease. This might be a cause of early blackening and the lack of flower opening. In previous studies using a large range of cut flowers, it was found that an exogenous carbohydrate supplementation is adequate to delay senescence. Many floral preservative solutions containing sucrose and a germicide as these are considered essential to extend the vase life of cut flowers (Halevy and Mayak, 1981). These preservatives also promoted flower bud opening, the lack of which occurs in several cut flowers such as roses (Goszczynska et al., 1990; Kuiper et al., 1995; Figueroa et al., 2005), tuberose (Hutchinson et al., 2003), and gladiolus (Marousky, 1968; Mayak et al., 1973).

The current study tested the hypothesis that low sugar levels in the petals account for the lack of bud opening and the early petal blackening in bud-cut lotus flowers.

Materials and methods

Plant materials

Flower buds of lotus (*N. nucifera* Gaertn., cv. Saddabutra, most likely identical to cv. Album Plenum) were harvested in the morning. The buds were picked at their normal commercial stage, that is, with the floral buds still fully closed but expected to open in

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about a day, if they had not been picked. Workers walked in the water of the lotus pond, which stood about half a metre deep, or collected stems by boat. Stems were broken under water, close to their junction with the rhizome. After harvest, the stems were held dry for at most 15 min or were immediately placed in purified water (tap water, after passing through a reverse osmosis apparatus). The stem length at harvest varied from 50 to 70 cm. Other flowers were harvested at slightly later stages, when the flower were about to open or had already opened, and the tip of the petals showed a gap of about 0.5–1.0 cm. Stems placed in purified water were brought to the laboratory within about 1 h of harvest. In the laboratory, the stems were recut in the air, to a length of 30 cm, then were placed individually in glass jars with purified water. The stems were held in a temperature-controlled room at 25 °C, about 70% relative humidity, and, unless otherwise indicated, natural light was supplemented with light from tungsten lamps (light from about 07.00 am to 07.00 pm; photon flux density 15 μmol/m^2/s).

Bud opening and petal blackening

The opening of floral buds was determined by measuring the maximum diameter of the gap at the tip of the petals, using a ruler. Abscission of petals was determined by measuring the time until half of all petals of a flower had fallen. Petal blackening was assessed by the time until half of the petals on a flower showed black patches. Observations were made daily at 09.00–10.00 am.

Levels of free carbohydrates

The soluble sugar content was determined using the method of Reyes et al. (1982), with slight modification. One gram fresh weight of lotus petal was ground with a cooled mortar and pestle. Five ml of 80% ethanol were added after which the mixture was incubated in a water bath at 80°C for 30 min. The extraction mixture was filtered through four layers of cotton cloth, then through a nylon membrane filter (0.45 μm pore size). The filtrate was freeze-dried under vacuum, re-dissolved in 5 mL of double deionized water and filtered through a nylon membrane filter (0.22 μm pore size). The soluble sugar content were determined and quantified by high performance liquid chromatography using a C18 column (Sugar Pak I; Waters; Milford, MA, USA) and a refractive index detector (RID-10A; Shimadzu; Kyoto, Japan). The filtered extract and 50 mg/L of calcium ethylenediamine tetraacetate (CaEDTA; Merck; Darmstadt, Germany) was pumped through the column at a flow rate of 0.5 mL/min and the temperature was adjusted to 90°C. Pure standards of sucrose (Merck; Darmstadt, Germany), D-glucose (BDH; Leuven, Belgium), and D-fructose (BDH; Leuven, Belgium) were identified by their retention times and were quantified by applying a range of known concentrations.

Sugar levels were determined in the outer, greenish petals, of stems cut at stage 5 (Fig. 1) and in the inner, white petals, in stems cut at later stages of development. In intact plants, sugar levels were determined at various stages of development, both in the outer and in the inner petals.

Inclusion of chemicals in the vase water

Sucrose, glucose, fructose, and 8-hydroxyquinoline sulphate (8-HQS) were obtained from Merck (Leuven, Belgium), BDH (Leuven, Belgium), BDH and Sigma–Aldrich (St Louis, MO, USA), respectively. Chemicals were included in the vase water at the onset of vase life and were not replenished. Glucose, fructose, or sucrose were applied at 5, 10, 15, 20, 25, 50 and 100 g/L, together with 200 mg/L 8-HQS as an antimicrobial compound. The 8-HQS was also tested separately.

Statistical analysis

All experiments in which flower opening, petal blackening, or petal abscission was measured used 8–10 flowers per treatment in a completely randomized design. Data of sugar treatments were tested using analysis of variance and an *F*-test at $p \leq 0.05$. Biochemical analyses used three biological replications, and mean values of treatments were analysed by the least significantly difference. Most of the experiments were repeated at a later date.

Results

Developmental stages of floral buds; effect of sugar feeding

The developmental stages of the floral buds and open flowers, as used in the present experiments, refer to flowers that remained attached to the plant. The identified stages are shown in Fig. 1 and are based on diameter classes.

Exogenously applied sugars might alleviate the effects of a lack of endogenous sugars, whereby some sugars might be taken up or used more efficiently than others. Glucose, fructose or sucrose were included in the vase solution at a range of concentrations in order to be able to find the optimum one. Sugars were applied together with 200 mg/L 8-HQS as an antimicrobial compound. The 8-HQS at 200 mg/L had no effect on the time to petal blackening or on flower opening (Fig. 2). The sugar treatments did not delay petal blackening, except in a few tests in which blackening in the controls was found extremely early (already by day 3). When applied at 50 g/L, each of the sugars had no effect, but rather hastened blackening, depending on the experiment. Petal blackening was invariably hastened when sugars were applied at 100 g/L (Fig. 2).

The sugar treatments had no effect on bud opening, either in flowers harvested when the buds were still tightly closed or if

Fig. 1. Stages of bud and flower development of the sacred lotus (*Nelumbo nucifera* spp. nucifera) cv. Saddabutra, as defined in the present paper. The stages were based on bud and flower diameter in attached buds and flowers. Buds at stage 1 had a diameter 2.0–2.5 cm, stage 2 a diameter of 3.0–3.5 cm, stage 3 a diameter of 4.0–4.5 cm, etc. The buds that were about to open were called stage 5 (diameter 6.0–6.5 cm), which is the commercial cutting stage. Flowers that had just opened (having an opening at the petal tips) were called stage 6 (diameter 7.0–7.5 cm). Flowers that had an opening of about 3–4 cm at their tips were at stage 7 (diameter 8.0–8.5 cm). The stage just before the abscission of the last (green) outer petals was called 8 (diameter 9.0–9.5 cm). The last of the (white) inner petals were about to abscise by stage 10 (diameter 11.0–11.5 cm).

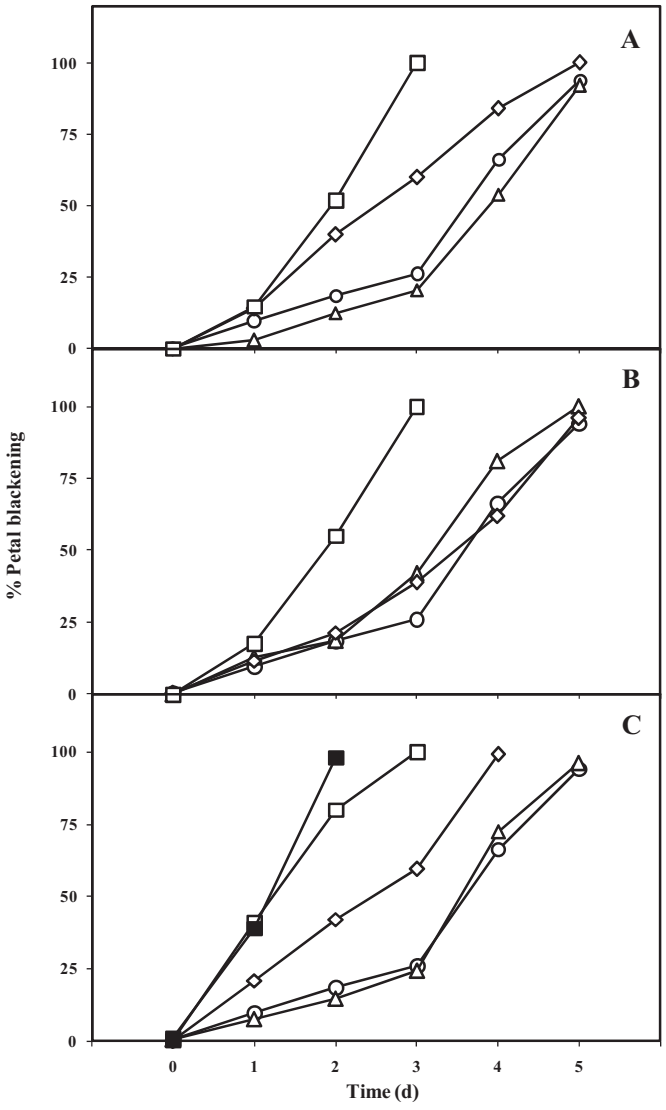

Fig. 2. Petal blackening in cut lotus flowers (*Nelumbo nucifera* spp. nucifera) cv. Saddabutra continuously placed in aqueous solution containing sugars at 25 °C. (A) Glucose. Control (○), 25 g/L glucose (△), 50 g/L glucose (◊), 100 g/L glucose (□). (B) Fructose. Control (○), 25 g/L fructose (△), 50 g/L fructose (◊), 100 g/L fructose (□). (C) Sucrose and mannitol. Control (○), 25 g/L sucrose (△), 50 g/L sucrose (◊), 100 g/L glucose (□), 25 g/L mannitol (■). All solutions contained 8-hydroxyquinoline sulphate, an antimicrobial compound, at 200 mg/L. This compound, applied alone, had no effect on blackening. Data are means of 10 replicate flowers.

harvested when the flower had already partially opened, as discussed in the following section (data not shown).

Effects of later harvest

A later harvest might be associated with higher levels of sugars or other compounds required for bud opening and for maintaining non-black petals. Experiments were carried out in the relatively cool season (November to February). Flower opening was compared when harvesting the closed buds (normal harvest stage) with buds that were slightly open at harvest, that is, showing an opening between the petal tips. When the opening between the petal tips was 2–3 cm at harvest, the flowers opened only slightly more and did so only toward the end of vase life (Fig. 3A). When the opening between the petal tips was 4–7 cm at harvest, some of the cut flowers opened to almost the size they would have attained if they had been left uncut (Fig. 3A).

Fig. 3B shows three examples. In the first example (top row of pictures; a), the petals at the time of harvest (day 0) had an opening between the petal tips of about 4 cm. In these flowers, a few green petals were still present by the time of harvest. By day 3 of vase life, all green petals had abscised. The outer flower surface was, therefore, white. On day 4 of vase life, the anther-like structures of the petaloid anthers had slightly blackened. By day 5, this blackening had increased and was considered to have ended vase life. The white petals showed no blackening on day 4 of vase life (Fig. 3B) and little blackening by day 5 (not shown). The flowers in the middle and bottom rows of pictures in Fig. 2B were harvested when the petal tips had opened still further. During vase life, the flower in the middle row in Fig. 3B showed relatively early abscission of all leafy floral parts and anthers (day 3). The flower in the lower row showed unacceptable excess opening already by day 2 of vase life. It also showed unacceptable blackening of the inner parts of the flower by day 3 (Fig. 3B). In this flower, abscission of all petals occurred on day 4 of vase life.

Free carbohydrates in the outer (green) petals of attached and cut flowers

As petal blackening during vase life (of flowers harvested at stage 5) was mainly visible on the outer greenish petals, the contents of glucose, fructose, and sucrose were compared in these petals, both in uncut and cut flowers (Fig. 4). In uncut flowers, the contents of glucose, fructose and sucrose remained similar from a young bud stage (stage 2) to the time of abscission of the green petals (stage 7; Fig. 4A). In flowers cut at the normal harvest stage (stage 5), and placed in water at 25 °C, the contents of glucose, fructose and sucrose rapidly decreased, in particular from day 0 to day 2 (Fig. 4B).

Free carbohydrates in the inner (white) petals of attached and cut flowers

When harvest took place, the flowers were already open (stage 8), most green petals had already fallen, and the remaining petals were about to fall. This explains why lotus flowers are white when in full bloom in the field.

The outer (green) petals showed hardly any growth during flower opening. The opening of the flowers in the field was mainly related to the growth of the inner, white petals (data not shown). Therefore the levels of free carbohydrates were also measured in white petals.

In uncut flowers, the contents of sucrose and glucose increased from stage 1 to 7 or 9, and then tended to decrease (Fig. 5A). The fructose levels increased from stage 7 to 10 (Fig. 5A). In flowers harvested at the commercial stage (stage 5) and placed in water in the climate room at 25 °C, the sucrose content decreased during vase life, from day 1 onward. The fructose content increased markedly from day 0 to day 4, then declined sharply on day 5 of vase life. No differences were observed in the glucose level (Fig. 5B).

In flowers harvested at stage 8 and placed in water in the climate room at 25 °C, the concentrations of glucose, fructose and sucrose in the white petals increased from the first day (Fig. 5C). In this experiment, about half of the petals that were present at stage 8 had abscised by day 3.

Discussion

In the outer (greenish) petals, the contents of glucose, fructose and sucrose remained about the same during a relatively long

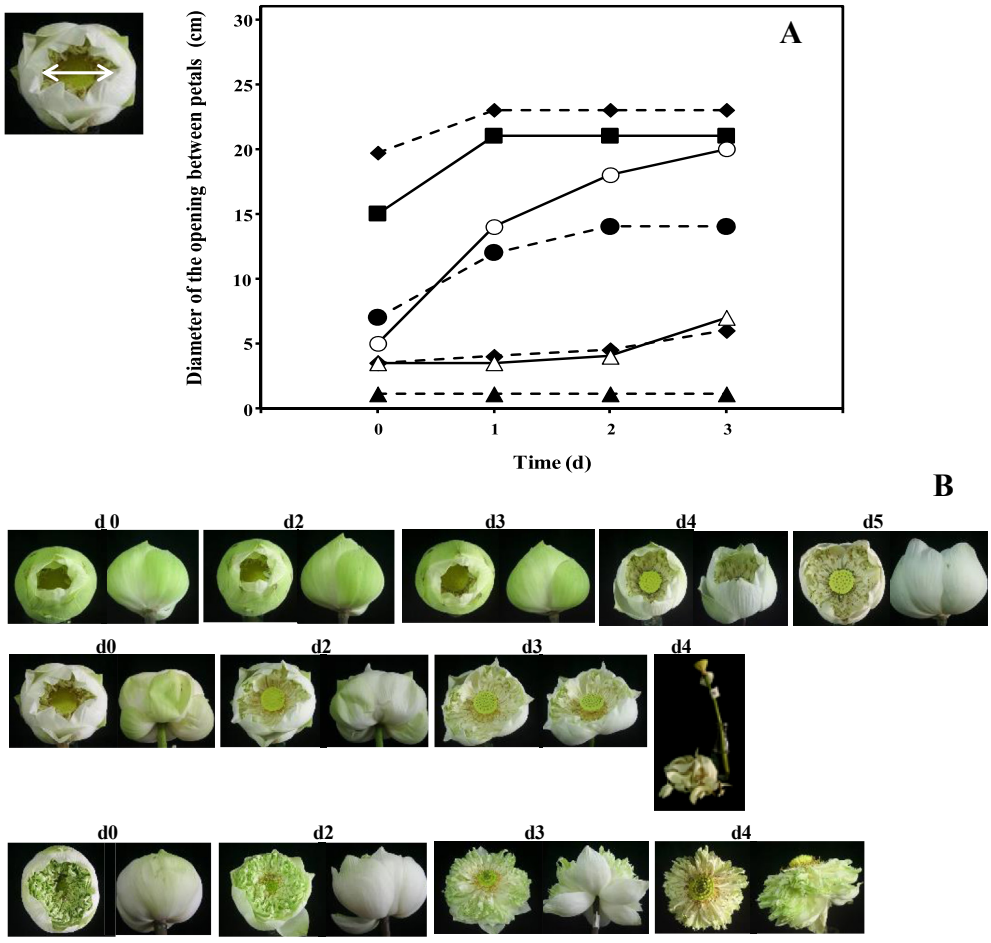

Fig. 3. Opening of cut flowers of the sacred lotus (*Nelumbo nucifera* spp. nucifera) var. Saddabutra. (A) Flowers were harvested at various stages of opening and were placed in water at 25 °C. The initial diameter of the opening between the petal tips; commercial harvest stage about 0.1 cm (▲ with dash line), 2.5 cm (Δ with solid line and ◆ with dash line), 5 cm (○ with solid line), 7 cm (● with dash line), 15 cm (■ with solid line) and maximum diameter about 18.7 cm (◆ with solid line), as indicated in the photograph on the left. Data refer to individual flowers. (B) Flower morphology during vase life. Flowers were cut at a later stage than the normal commercial harvest stage (stage 5), which is just before the petals separate at their top. Upper row of pictures: petals separated by about 2.5 cm at harvest. Middle row of pictures: petals separated about 6 cm at harvest. Lower row of pictures: petals separated by about 8 cm at harvest.

period of bud and flower development in non-cut plants. In floral buds that were cut at the commercial harvest stage (stage 5) and placed in water, the contents of these three sugars in the greenish petals decreased rapidly from day 0 of vase life. A lack of available sugars might be a reason for early petal blackening in these green petals.

In order to find out if low sugar levels were the cause of petal blackening and lack of flower opening, glucose, fructose or sucrose were included in the vase solution. In many other cut flowers, such a treatment has been reported to delay petal senescence and result in longer maintenance of bright flower colour; in several cut flowers tested, the inclusion of these sugars in the vase solution promoted bacterial growth and resulted in low water uptake due to a bacterial blockage in the xylem (van Doorn, 1997, 2012). Therefore, the current study tested sugars with 200 mg/L of 8-HQS, an antibacterial compound. Preliminary tests had shown that this concentration did not induce toxic symptoms and was effective against bacteria in a vase solution that contains sugars. The present data showed no effect of 8-HQS. Remarkably, there was also no positive effect on petal blackening from any of the sugars tested, in combination with 200 mg/L 8-HQS. This result strongly indicated that petal blackening in untreated, control, cut lotus flowers is not due to a lack of sugars. The vase life of lotus flowers is unique, as it is limited by petal blackening. This blackening is regulated by a set of controls that are not yet fully understood.

Previous experiments showed a delay of petal blackening caused by cytokinins and gibberellic acid in vase water (Imsabai and van Doorn, 2013). This means that the hormones apparently reached the petals, which indicates that sugars, which were applied in the same way, also reached the petals. Therefore, the lack of an effect was apparently not attributed to the lack of sugars in the petals, although it is not known how much of the sugars was transported into the petal cells.

When lotus flowers were held in an aqueous solution containing gibberellic acid (GA_3) combined with sugar, or in GA_3 without sugar, the vase-life in both treatments was the same (Netlak, 2013). This suggests that the increase in vase-life (due to late petal blackening) was solely due to the effect of GA_3.

As indicated above, cytokinins such as 6-benzylaminopurine (BA) and thidiazuron also delayed petal blackening (Imsabai and van Doorn, 2013). Cytokinins might possibly counteract the effects of endogenous ethylene. Similarly, in dahlia flowers, whose vase life was limited by ethylene, BA extended vase life, also when applied together with a compound releasing ethylene. This shows that BA acted, at least in part, as an ethylene antagonist (Shimizu-Yumoto and Ichimura, 2013). The effect of cytokinins in lotus

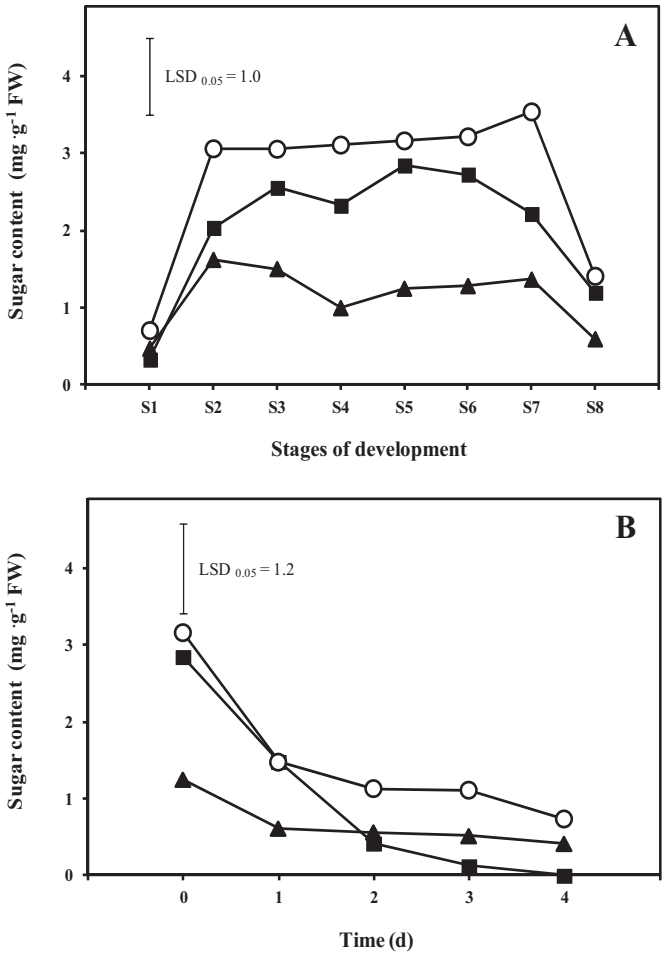

Fig. 4. Sucrose, glucose and fructose contents in the outer (green) petals of the sacred lotus (*Nelumbo nucifera* spp. nucifera) cv. Saddabutra. Stages of flower development as in Fig. 1(A) Intact plants (B) Cut flowers harvested at the normal harvest stage (stage 5) and placed in water at 25 °C. Sucrose (■), glucose (▲), fructose (○). Data are means of three replicate biological samples. Error bars show least significant difference at $p \leq 0.05$ level.

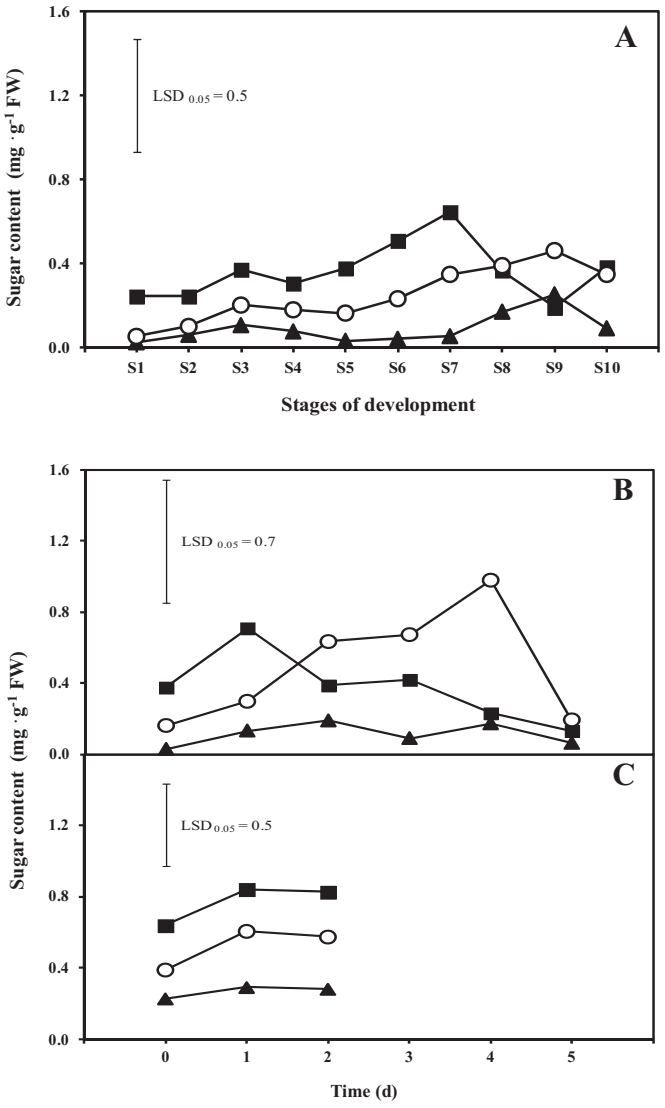

Fig. 5. Sucrose, glucose and fructose contents in the inner (white) petals of the sacred lotus (*Nelumbo nucifera* spp. nucifera) cv. Saddabutra. Stages of flower development as in Fig. 1(A) Intact plants. (B) Cut flowers harvested at the normal harvest stage (stage 5) and placed in water at 25 °C. (C) Cut flowers harvested at stage 8 and placed in water at 25 °C. Sucrose (■), glucose (▲), fructose (○). Data are means of three replicate biological samples. Error bars show least significant difference at $p \leq 0.05$ level.

might thus be explained, while that of gibbellic acid is still an enigma, although interactions between the two hormones, including an effect of gibberellic acid on ethylene sensitivity, are known at least during seed germination (Corbineau et al., 2014). Flower opening in cut lotus flowers was observed when buds were harvested at a slightly later stage than the normal harvest stage. It is not clear what physiological factors accounted for this effect. The opening of floral buds on the lotus seems to be mainly related to the growth of the inner (white) petals. Analysis of sugar contents in the petals of cut buds did not indicate that low levels of free carbohydrates accounted for the lack of flower opening. Treatments with sugars in the vase solution did not promote bud opening of buds cut at stage 5 of development. The lack of opening, therefore, does not seem to be due to a lack of available sugars in the inner petals. An additional argument against the role of sugars in the petals is that in buds cut at stage 8 of development, which opened during vase life, the levels of sugars in the white petals were not much different from buds cut at stage 5.

In lotus buds cut at stage 5 of development, the contents of glucose, fructose and sucrose in the outer (green) petals decreased from day 0 of vase life. This might be a reason for early petal blackening in these green petals and the lack of flower opening. However, treatments with glucose, fructose or sucrose in the vase solution had no effect on petal blackening, except when the time to petal blackening in the controls was short (less than 4.1 d, which was rare in these experiments). The sugar treatments also did not promote flower opening. This suggests that in most experiments, a low endogenous sugar content was not the cause of early petal blackening or the lack of bud opening. The contents of glucose, fructose and sucrose in the inner (white petals) part also did not decrease during vase life, in flowers cut at stage 5. This indicated that sugars in these parts also did not seem to account for the lack of flower opening. Harvesting at a later phase of flower development resulted in flower opening, but this was not related to the sugar levels in the petals. Together, the data suggest that petal sugar levels do not explain the lack of bud opening and early petal blackening.

Conflict of interest

None declared.

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