

The impact of fruit temperature dynamics on heat stress tolerance of selected oil pumpkin genotypes

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Abstract: Fruit temperature is a key parameter for fruit growth and quality which is affected by climate, plant vigorosity, solar exposure and fruit thermal properties. In the present study, the variability in temperature dynamics of Styrian oil pumpkin fruits and selected interspecific hybrids involving *Cucurbita argyrosperma*, *C. moschata*, *C. pepo* was analysed in two different periods of hot weather. The temperatures were measured with thermistors on (a) attached fruits, (b) detached fruits exposed to the sun and (c) artificially black coloured fruits. The highest average temperatures were determined in the Styrian oil pumpkin, whereas the lowest temperatures were determined in genotypes with lighter fruit exteriors suggesting that those are less sensitive to heat stress conditions and may represent a good option for the improvements of adaptability to climatic changes. In order to combine lighter and harder pericarp, the most promising genotypes were crossed with wild *Cucurbita okechobeensis*. The histological analysis showed that *C. okechobeensis* was a good source of genes for obtaining a thicker sclerenchymatic layer within pericarp.

1. Introduction

Oil pumpkins play a significant role in human nutrition and health. The nutritional value of oil pumpkin seeds is based on high protein and antioxidant content, and high energy potential due to the high percentage of oil (Fruhirth and Hermetter, 2007; Sari *et al.*, 2008; Fokou *et al.*, 2009; Lelley *et al.*, 2009; Urbanek Krajnc *et al.*, 2016). During the last decade, oil pumpkin cultivation declined regarding productivity and quality due to the outbreaks of the *Zucchini yellow mosaic virus* (ZYMV), extremely high temperatures, radiation stress and prolonged periods of drought (Lelley *et al.*, 2009; Seebold *et al.*, 2009; Gong *et al.*, 2013). Years 2013 and 2015 have been excessively hot and we have seen serious problems in fruiting pumpkins related to weather conditions especially high day/night temperatures and drought stress (Yavuz *et al.*, 2015).

Heat stress depends on intensity, duration, and rate of increase in temperature. The extent to which it occurs, in specific climatic zones, depends on duration and level of high temperatures occurring during

the day and/or the night. In general, a transient elevation in temperature, 10-15°C above ambient, is considered as heat stress (Wahid *et al.*, 2007). It causes an array of morpho-anatomical, physiological and biochemical changes in plants, which affect plant growth and development and may lead to a drastic reduction in economic yield. On the morphological level, high temperature can cause considerable pre- and post-harvest damages such as scorching of leaves and stems, sunburns on leaves, stems and fruits, leaf senescence and wilting, shoot and root growth inhibition, fruit discoloration and damage, and reduced yield (Wahid *et al.*, 2007; Ara *et al.*, 2015; Johnson, 2015). The physiological changes caused by high temperatures include the negative effect on photosynthesis, respiration, water relations, and modulated levels of hormones and primary and secondary metabolites. On the biochemical and sub-cellular level the direct injuries due to high temperatures include protein denaturation and aggregation, disorganization of cytoskeleton and increased fluidity of membrane lipids. Indirect or slower heat injuries include inactivation of enzymes in chloroplast and mitochondria, inhibition of protein synthesis, protein degradation and loss of membrane integrity. These injuries eventually lead to starvation, inhibition of growth, reduced ion flux, production of toxic com-

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pounds and reactive oxygen species (ROS), which coincide with increased synthesis of antioxidants and activity of antioxidant enzymes. Furthermore, enhanced expression of a variety of heat shock proteins and other stress-related proteins constitute major plant responses to heat stress (Iba, 2002; Howarth, 2005; Wahid *et al.*, 2007; Ara *et al.*, 2013 a, b, 2015).

During the ontogenetic development of plants, flowering and fruit set are the most sensitive stages; fruit set of pumpkins is affected at day/night temperatures above 26/20°C and is severely affected above 35/26°C (Schrader *et al.*, 2004, 2011; Saudreau *et al.*, 2011; Lei *et al.*, 2014; Johnson, 2015). The decrease in yield is due to the rapid decrease of photosynthesis, which reduces the amounts of sugars and other storage products that can go into fruits and developing seeds. On the other hand, hot night temperatures lead to greater cell respiration. High temperatures can also cause increased developmental disorders in fruiting vegetables due to the reduced pollen production leading to a reduction in fruit and seed sets, smaller pods, and split sets. Another effect of heat stress in many plant species is induced sterility when heat is imposed immediately before or during anthesis (Siddique *et al.*, 1999; Wahid *et al.*, 2007).

Theoretical and experimental evidence shows that fruit temperature can be 10°C higher than the air temperature under sunny conditions (Schrader *et al.*, 2004; Racskó *et al.*, 2005; Schrader, 2011; Lei *et al.*, 2014). Sunburn of fruits is a surface injury caused by solar radiation which, during the initial phase, results in a light corky layer, golden or bronze discolouration. The damage occurs mainly in the surface and subsurface layers. There are two types of sunburn damage which may have effects on fruits and fruiting vegetables. The first, sunburn necrosis, appears due to the thermal death of cells on the sun exposed side of the fruit; cell membrane integrity is lost and cells start leaking their contents (Schrader, 2011; Johnson, 2015). The critical fruit tissues' temperatures for sunburn necrosis vary with the type of fruit. The fruit surface temperature (FST) threshold for sunburn necrosis for cucumbers and pumpkins is between 37 and 42°C (Rabinowitch *et al.*, 1983, 1986; Ara *et al.*, 2013 a, b; 2015). The second type of sunburn injury is sunburn browning, which is caused by the combination of high FST and high solar radiation. It causes degradation of photosynthetic pigments resulting in yellow spots on the sun-exposed side of the fruit and occurs at a temperature about 5°C lower than sunburn necrosis (Schrader, 2011; Johnson, 2015).

Plants have three major ways in which they dissipate excess heat: (1) long-wave radiation, (2) heat convection into the air and (3) transpiration. If transpiration is interrupted by stomatal closure due to water stress, inadequate water uptake or other factors, a major cooling mechanism is not functioning. This will cause internal leaf/fruit temperatures to rise. Without transpiration, the only way that plants can reduce heat is by heat radiation back into the air or wind cooling. Under high temperatures, radiated heat builds up in the atmosphere around plants, limiting further heat dissipation (Wahid *et al.*, 2007; Schrader, 2011; Johnson, 2015).

The adverse effects of high air and soil temperatures, and the high levels of solar radiation can be mitigated by developing plant genotypes with improved thermotolerance. Some attempts to develop heat-tolerant genotypes via conventional breeding protocols have been successful (Ehlers and Hall, 1998; Camejo *et al.*, 2005). Breeding of cultivated cucurbits was mainly focused in combining good attributes of *C. moschata* and *C. maxima*. (Balkaya and Karaagac, 2005; Balkaya *et al.*, 2009, 2010 a, b, 2011; Balkaya and Kandemir, 2015). It is well known that *C. moschata* is best adapted to hot climate and is successfully cultivated in tropical and subtropical regions (Balkaya *et al.*, 2010 a, b; Balkaya and Kandemir, 2015). Recently, a study was conducted to determine the extent of heat tolerance of newly developed interspecific squash hybrid named as 'Maxchata' compared to its parents *C. maxima* and *C. moschata* (Ara *et al.*, 2013 a, b, 2015) under three different temperature regimes. Results showed that various gas exchange and photosynthetic attributes dropped significantly with increasing temperature, while intercellular CO₂ concentration increased showing the nonstomatal limitations. These trends were more abrupt in *C. maxima*, reflecting that *C. maxima* was the most susceptible, while 'Maxchata' showed intermediate response. *C. moschata* had the best photosynthetic attributes to sustain the heat regimes (Ara *et al.*, 2013 a, b). The ultramorphological, biochemical, and transcriptional analyses gave similar results. The electron microscopy highlighted the maximum degradation of the leaf ultrastructure of *C. maxima*. *C. moschata* and 'Maxchata' exhibited lower degree of subcellular injury upon heat exposure. The antioxidant enzyme activities and their expression were found to be highest in *C. moschata*, moderate in 'Maxchata', and lowest in *C. maxima* (Ara *et al.*, 2013 a, b; 2015). The authors concluded that the interspecific hybridization with *C. moschata* might significant-

ly contribute to heat tolerance (Ara et al., 2013 a, b, 2015).

The presented study is associated with creating heat tolerant pumpkins characterised by lighter exocarp. The work began in 1996 and is based on a modified recurrent selection approach. Its aim is to create cultivars characterised by bushy growth, resistance to all major pests and diseases, tolerance to drought and high temperatures, and large and thick seeds having thin seed coats and high concentrations of high quality oil.

The basic population (i.e., population of the cycle-0) was established by inter-crossing all available sources of genes (i.e., numerous local and commercial cultivars, populations and hybrids of *C. pepo*, following the semi-diallel scheme). In 1997, the most valuable progenies were planted in New Caledonia, at the CIRAD centre near Pouembout (South Pacific). Due to the favourable semi-tropical climate, it was possible to execute three cycles per year. The problems, however, were seed germination within fruits and rotting fruits due to overheating. As the genetic resources within *C. pepo* were found to be insufficient for overcoming these problems, it was decided to incorporate interspecific hybridisation and change the exterior fruit colour. The main sources of genes for lighter fruit exterior were *C. argyrosperma* var. *argyrosperma* and *C. moschata*. *Cucurbita argyrosperma*, which was the main source of genes for whitish exocarp and was also used as a genetic bridge between *C. pepo* and *C. moschata*. Another interesting trait obtained by interspecific crosses was dark yellow fruit exterior which was associated with the same colour of mesocarp. Some years later, a wild species *C. okechobeensis* (Small) L. H. Bailey was added to the hybridisation programme, in order to improve the resistance to viruses and harder pericarp. The interspecific hybrids included in this study were indirect progenies developed within the recurrent selection programme which involved intra- and inter-population crosses, self-pollinations, and back-crosses.

The progress in breeding for heat stress tolerance strongly depends upon understanding the genetic and physiological mechanisms associated with stress tolerance of the whole plants well as at the molecular and cellular levels. Our study involved thin-coated seed pumpkins with lighter exocarp because they were considered to have the highest level of tolerance to high air temperatures and high levels of solar radiation during summer. In order to investigate their tolerance to heat stress, pericarp tissue temperature

profiles were monitored on the exocarp surface, as well as in the meso- and endocarp of fully developed attached and detached fruits during two different periods of hot weather.

2. Materials and Methods

Plant material

Four different plant materials with thin coated seed were used in the study: (1) Styrian oil pumpkin *Cucurbita pepo* subsp. *pepo* var. *styriaca* (O), (2) progeny with whitish fruit derived from the cross *C. pepo* (non-lignified seed coat, oil type) × *C. argyrosperma* var. *argyrosperma* (O/A), (3) a progeny derived from crosses involving *C. pepo* (non-lignified seed type, oil type), *C. argyrosperma* and tropical *C. moschata* characterised by yellow fruits (A/Mo × O/A) and (4) a three-species hybrid involving *C. pepo* (non-lignified seed coat, oil type), *C. argyrosperma* var. *argyrosperma* and *C. okechobeensis* (Oke × O/A) (Table 1, Fig. 1).

Table 1 - List of key plant materials with short explanations of abbreviations

Plant material	Abbreviations
<u>Species</u>	
<i>Cucurbita argyrosperma</i> var. <i>argyrosperma</i>	A
<i>Cucurbita moschata</i>	Mo
<i>Cucurbita pepo</i> subsp. <i>pepo</i> var. <i>styriaca</i>	O
<i>Cucurbita okechobeensis</i>	Oke
<u>Interspecific hybrids</u>	
Material obtained from the cross <i>C. pepo</i> (non-lignified seed coat, oil type) × <i>C. argyrosperma</i> var. <i>argyrosperma</i> , followed by several seasons of intrapopulation recombination and selection	O/A
Three-species hybrid involving <i>C. argyrosperma</i> (used as a genetic bridge), <i>C. moschata</i> and <i>C. pepo</i> (non-lignified seed coat, oil type), after several seasons of intrapopulation crosses and selection	A/Mo × O/A
A three-species hybrid involving <i>C. pepo</i> (non-lignified seed coat, oil type), <i>C. argyrosperma</i> var. <i>argyrosperma</i> and <i>C. okechobeensis</i>	Oke × O/A

The temperatures were measured on (a) attached fruits (1st period, 27th July to 5th August 2012), (b) detached fruits exposed to the sun (2nd period, 1st to 11th September 2012) and (c) artificially black coloured fruits (on both periods). The fruits were exposed to sunlight due to loss of foliage caused by drought stress and diseases. For each experiment, three fruits of each studied material were used.

Fruit temperature measurements

The temperatures were measured during two different periods of hot weather, between the 27th July

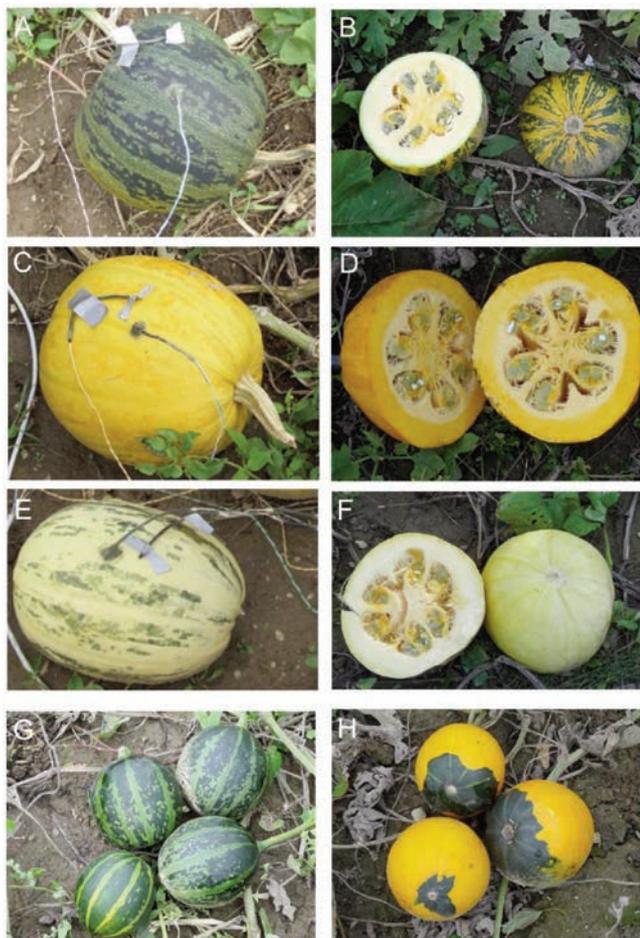


Fig. 1 - Fruits of selected genotypes with attached sensors, which were selected for lighter and harder fruit exterior and thin coated seeds for: A, B) Styrian oil pumpkin (O). C, D) Three-species hybrid involving *C. argyrosperma* var. *argyrosperma* (used as a genetic bridge), *C. moschata* and *C. pepo* (non-lignified seed coat, oil type), after several seasons of intrapopulation crosses and selection (A/Mo × O/A). E, F) Genotype with lighter (i.e., whitish, white-green) fruits material obtained from the cross *C. pepo* (non-lignified seed coat, oil type) × *C. argyrosperma*, followed by several seasons of intrapopulation recombinations and selection (O/A). G) *C. okeechobeensis*. H) A three-species hybrid involving *C. pepo* (non-lignified seed coat, oil type), *C. argyrosperma* and *C. okeechobeensis* (Oke × O/A).

to 5th August 2012 and 1st to 11th September 2012. During both periods, three pumpkins of each progeny (O, O/A, A/Mo × O/A) were chosen and the thermistors were placed on exocarp the sun-exposed side, as well as inserted in meso- (2 cm deep) and endocarp (10 cm deep) (Fig. 1A, C, E). The temperatures were recorded every 15 seconds and stored as 10-min averages. The data represented in figures 3-6 show a diurnal and maximum day temperature as average of three fruits out of each progeny.

In order to measure temperatures, 32 BETA-THERM 10K3A542I thermistors connected to a Campbell's CR1000 datalogger (Campbell Scientific

Inc., Logan, UT, USA) were used. The data logger recorded the current times for each iteration supply voltage and analogue input voltage. The thermistors' readings were performed sequentially as they were all connected to the same ADC converter through a multiplexor; so only one of the thermistors was connected to the converter at a time.

The selected thermistors had accuracies of 0.2°C and were of negative-temperature-coefficient (NTC) type, which meant that their resistances decreased with the increases of temperature. As the data logger was unable to read the resistance of the thermistor directly, the thermistors were connected in a form of voltage divider, with an additional resistor with 1 KΩ±0.1% fixed resistance. As the resistive responses of the thermistors were nonlinear, the measured temperatures were calculated according to the temperature table from the datasheet (BETHATERM).

The results are represented by mean values (N=3), and were statistically evaluated by one-way analysis of variance (ANOVA), using the SPSS 21 software (SPSS 21 software, SPSS Inc., Chicago, IL, USA). Significant differences between mean values were determined using the post-hoc Duncan test. Significant differences ($\alpha < 0.05$) between means were indicated by different letters.

Air/soil measurements

Furthermore, air/soil temperatures and relative humidity were measured on the sun-exposed side and within the canopy (5 and 20 cm below soil surface, on soil surface as well as 10 and 20 cm above soil surface using 215 and 107 Temperature Probe sensors (Campbell Scientific Inc., Logan, UT, USA). The temperatures were recorded every 15 seconds and stored as 10-min averages.

Histochemical evaluation of pericarp

From each studied material, 3-6 fruits were taken for histological evaluation. Out of each fruit six pieces (diameter 8 mm) of pericarp were sampled on equator of the fruit positioned around the pumpkin in regular intervals. The pieces were cut with a cryotom in order to evaluate the size of the lignified cell layer. An Olympus microscope (Provis AX 70) with a 100 W mercury arc lamp was used to take analogue images with a 3-chip-colour video camera (Sony DXL 950 P, 3 CCD). Fluorescence images were obtained through an UplanFI 40x dry objective (n.a., 0.75), a PlanApo 60x oil immersion objective (n.a., 1.40), and an UplanApo 100 x oil immersion objective (n.a., 1.35). Lignified cells were visualised using an Olympus filter set (U-MWU) with 330-385 excitation and 420 nm emission.

3. Results and Discussion

Our research was based on the hypothesis that interspecific hybridization aimed in creating lighter exocarp, thicker hypodermal sklerenchyma layer and cuticle would reduce the heat load on the fruit.

Air/soil measurements

During the first period of measurements (Jul.-Aug.), on the sun-exposed sides, the highest temperatures were registered on the ground surface reaching 45°C, whereas 20 cm above ground the maximum daily air temperatures were about 38°C. On the sides shaded by a canopy, the ground temperatures remained cooler and more stable, varying between 18 during the nights and 32°C on the hottest days. Twenty cm above ground, the air temperatures reached 43°C. The underground temperatures (20 cm deep) remained stable with 20-22°C day/night variation (Fig. 2 A).

Additionally, the ground temperatures were also measured within the proximities of the studied pumpkins. In the proximity of Styrian oil pumpkins, from the 30th of July onwards, they increased from 27°C (daily maximum) to more than 47°C on the 1st August, and remained above 40°C for the next five days (Fig. 3 A, B). The maximum day ground temperature in the proximity of white pumpkins were similar, reaching 45°C during the first three days of August (Fig. 3 E, F).

At the beginning of September, the minimum night temperature was 10°C. One day, on the 7th September, it was particularly hot and the air temperature 20 cm above ground, on the sun-exposed side, reached 45°C, whereas the temperature of the ground surface reached 58°C. A similar situation was observed within the canopy. At 20 cm height, the temperature reached 45°C (Fig. 2 B).

On attached Styrian oil pumpkins (1st period), the maximum day temperatures measured on the exocarp varied between 38 and 50°C. In mesocarp the temperatures ranged between 32 and 51°C. Endocarp was more or less 2°C cooler than mesocarp (Fig. 3 A, B). On detached Styrian oil pumpkins (2nd period) the maximum day temperatures of exocarp varied between 20 and 54°C, whereas mesocarp heated up above the temperature of exocarp to 61°C on the hottest day (7th September), on later days, the maximum day temperatures were around 45°C (Fig. 4 A, B).

In the 'yellow fruit' pumpkin material (A/Mo × O/A), the temperatures of exocarp ranged between

42°C and 50°C on attached fruits during the first period and 17°C and 50°C on detached fruits during the second period of hot weather. The temperatures of endocarp varied between 33°C and 42°C during the first period, whereas during the second period the temperatures of mesocarp of detached pumpkins ranged between 16°C and 45°C (Fig. 3 C, D, Fig. 4 C, D).

'White fruit' pumpkin material (O/A) was characterised by the lowest exocarp temperatures, which ranged between 30°C and 41°C during the first period, whereas during the second period the temperatures ranged between 17°C and 44°C. The mesocarp temperatures varied between 35 and 44°C, the endocarp temperatures were more or less 2°C above the mesocarp temperatures. During the second period, where temperatures were measured on detached fruits, the average temperatures of mesocarp were

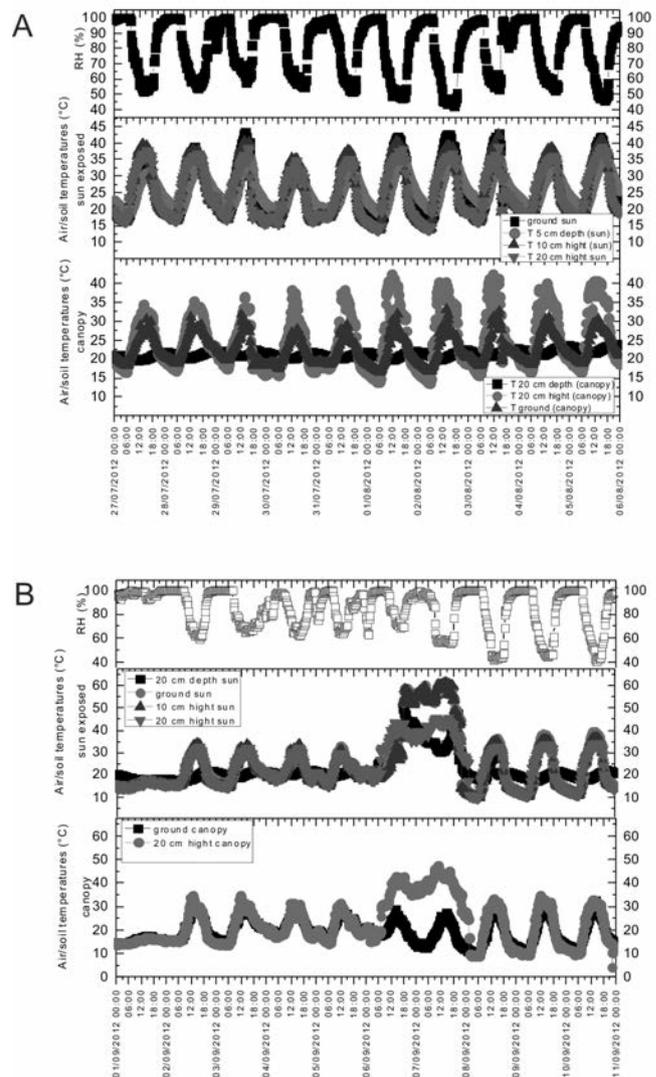


Fig. 2 - Relative humidity and the average air/soil temperatures measured within canopy and on sun exposed side during the period between 27 July-6 August 2012 (A) and between 1-11 September 2012 (B).

generally 2°C lower when compared to exocarp but the fruit flesh heated up to 56°C on the extremely hot days of the 6th and 7th September, similarly as was observed in the Styrian oil pumpkin (Fig. 4 A, B, E, F, Fig. S1 see supplementary material).

Transpiration appeared to be vital for maintaining optimal growth temperatures in growing plants. Detached fruits, however, lacked the protective effects of transpiration, and direct sources of heat, such as sunlight, can rapidly elevate the internal fruit

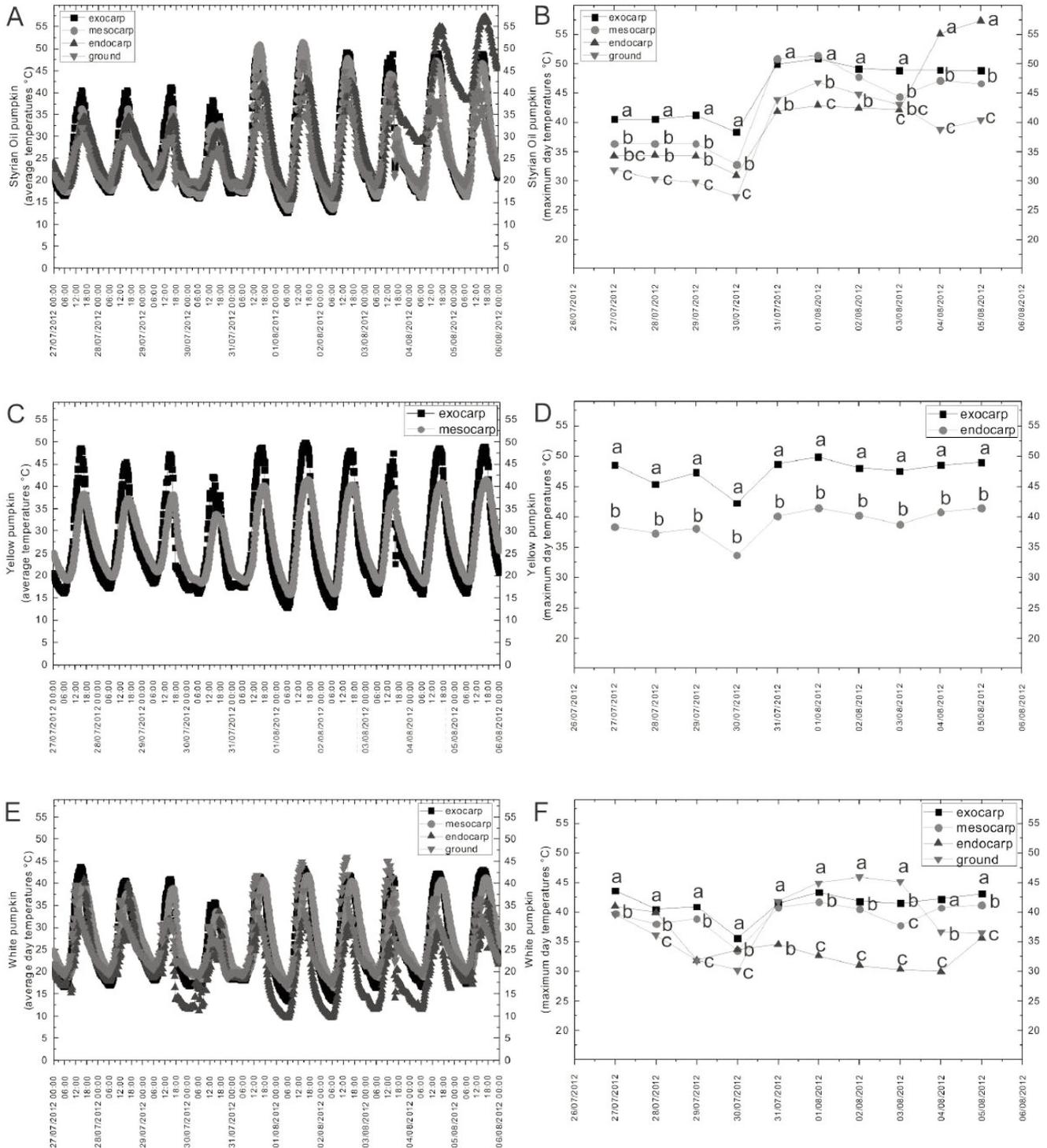


Fig. 3 - Average and maximum day temperatures measured in exocarp, mesocarp, endocarp of attached pumpkins and ground in the period between 27. July - 6. August 2012: A, B) Styrian oil pumpkin (O). C, D) Genotype with yellow fruits involving *C. argyrosperma*, *C. moschata* and *C. pepo* (A/Mo × O/A). E, F) Genotype with white fruits obtained from the cross *C. pepo* (non-lignified seed coat, oil type) × *C. argyrosperma* (O/A).

temperatures to above that of exocarp and towards the thermal death points of their cells. In case of Styrian oil pumpkin these lead to localised bleaching and necrosis (sunburn or sunscald). Similarly, Rabinowitch *et al.* (1983, 1986) reported that

detached cucumbers (*Cucumis sativus* L.) and peppers (*Capsicum annuum* L.) had significantly higher surface temperatures and more serious sunburn injuries than attached fruit.

Comparing the measured results of the different

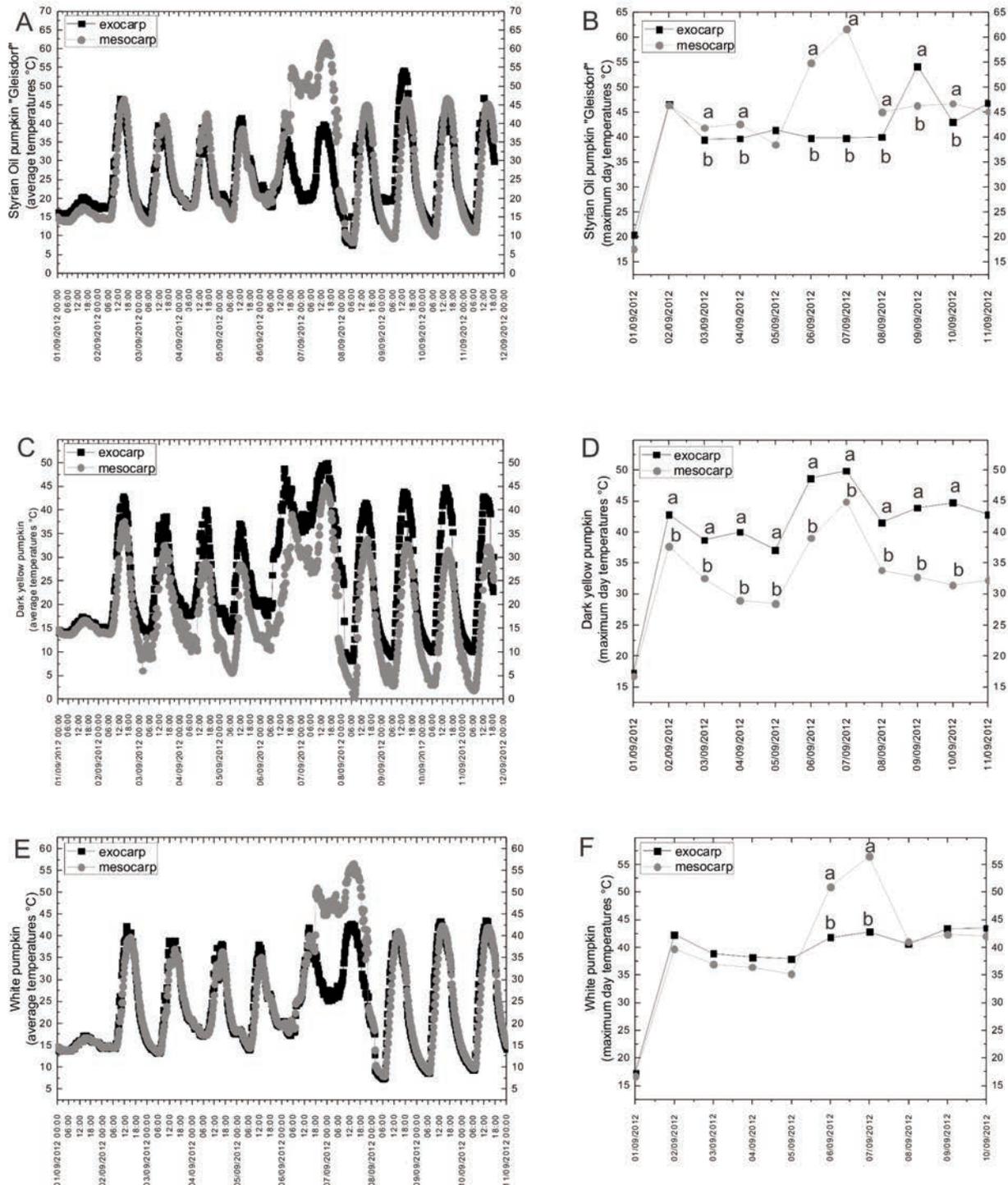


Fig. 4 - Average and maximum day temperatures measured in exocarp and mesocarp of detached pumpkins in the period between 1.-11. September 2012: A, B) Styrian oil pumpkin (O). C, D) Genotype with yellow fruits involving *C. argyrosperma*, *C. moschata* and *C. pepo* (A/Mo × O/A). E, F) Genotype with white fruits obtained from the cross *C. pepo* (non-lignified seed coat, oil type) × *C. argyrosperma* (O/A).

varieties on attached fruits, we can conclude that Styrian oil pumpkin was characterised by the highest temperatures within all three tissues (Fig. S2), followed by yellow genotype, whereas white genotype had the lowest temperature, confirming the hypothesis that varieties with lighter fruit colour are less heated up. Our results of the experiment on detached fruits, however, suggest that the most tolerant is A/Mo × O/A which is a progeny derived from the interspecific cross involving the Styrian oil pumpkin, *C. argyrosperma* and *C. moschata*. It is well known that *C. moschata* is best adapted to hot climate and is successfully cultivated in the tropical and subtropical regions (Balkaya *et al.*, 2010 a, b; Balkaya and Kandemir, 2015). Heat tolerance of *C. moschata* can be very useful in breeding involving interspecific hybridisation. One of the successful examples, mentioned earlier in the text, is interspecific hybrid of

squash named as ‘Maxchata’. The authors concluded that the interspecific hybridization involving *C. moschata* might significantly contribute to heat tolerance (Ara *et al.*, 2013 a, b; 2015).

In order to test further the resistance of selected genotypes to heat stress, the selected pumpkins were sprayed with black colour and exposed to the sun in order to induce bleaching by the excess heat. The exocarp of the black coloured Styrian oil pumpkin fruit heated up to 52°C whereas the mesocarp temperature rose up to 43°C in the afternoon hours between 3 and 4 p.m. during both periods. When analysing the daily temperature curves of attached pumpkins during the July-August period, a depression in the curve was observed at around 12 a.m., reflecting a higher transpiration of fruits in response to heat stress; later, a rapid increase in temperature, may indicate stomata closure (Fig. 5A). It is well-

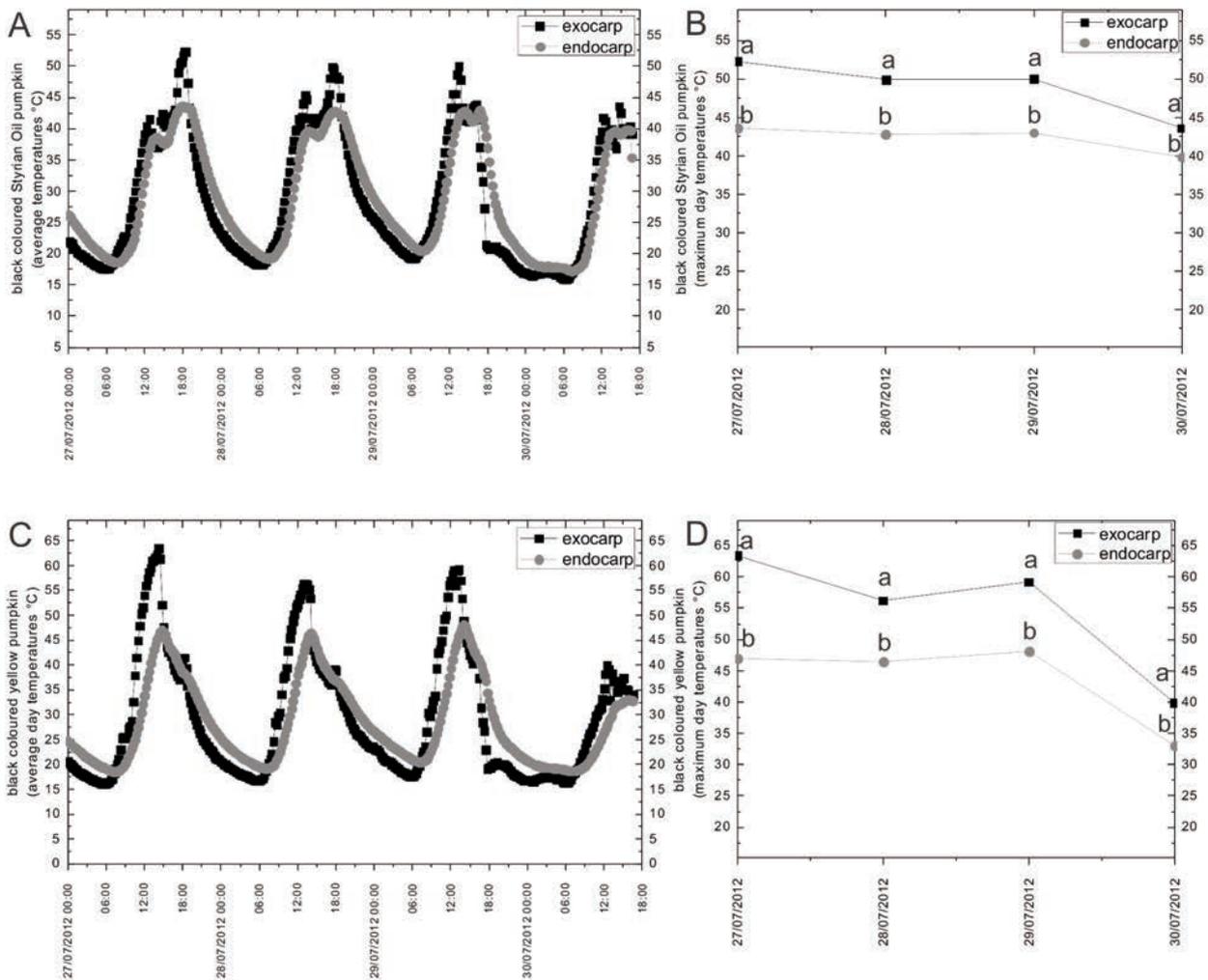


Fig. 5 - Average and maximum day temperatures measured in exocarp and endocarp of black coloured attached pumpkins measured in the period between 27 July- 6 August 2012: A, B) Styrian oil pumpkin (O). C, D) Genotype with yellow fruits involving *C. argyrosperma*, *C. moschata* and *C. pepo* (A/Mo × O/A).

known that if transpiration is interrupted by stomatal closure a major cooling mechanism is lost and the internal fruit temperatures raise (Wahid *et al.*, 2007; Johnson *et al.*, 2015). The temperatures of coloured fruits were measured for three days since a general collapse and tissue death of the inner pericarp layers was observed later on. The fruits were too much damaged by sunburn and measurements of tempera-

tures no longer made sense. In the September period, when the experiment was performed on detached fruits, the exocarp of Styrian oil pumpkin heated up to 51°C, similarly as on attached fruits. Mesocarp temperature raised to a higher level on detached fruits (61°C) in comparison to attached fruits (43°C) (Fig. 6A, Fig. 6B, Fig. S2).

Interestingly, during the 1st experiment on

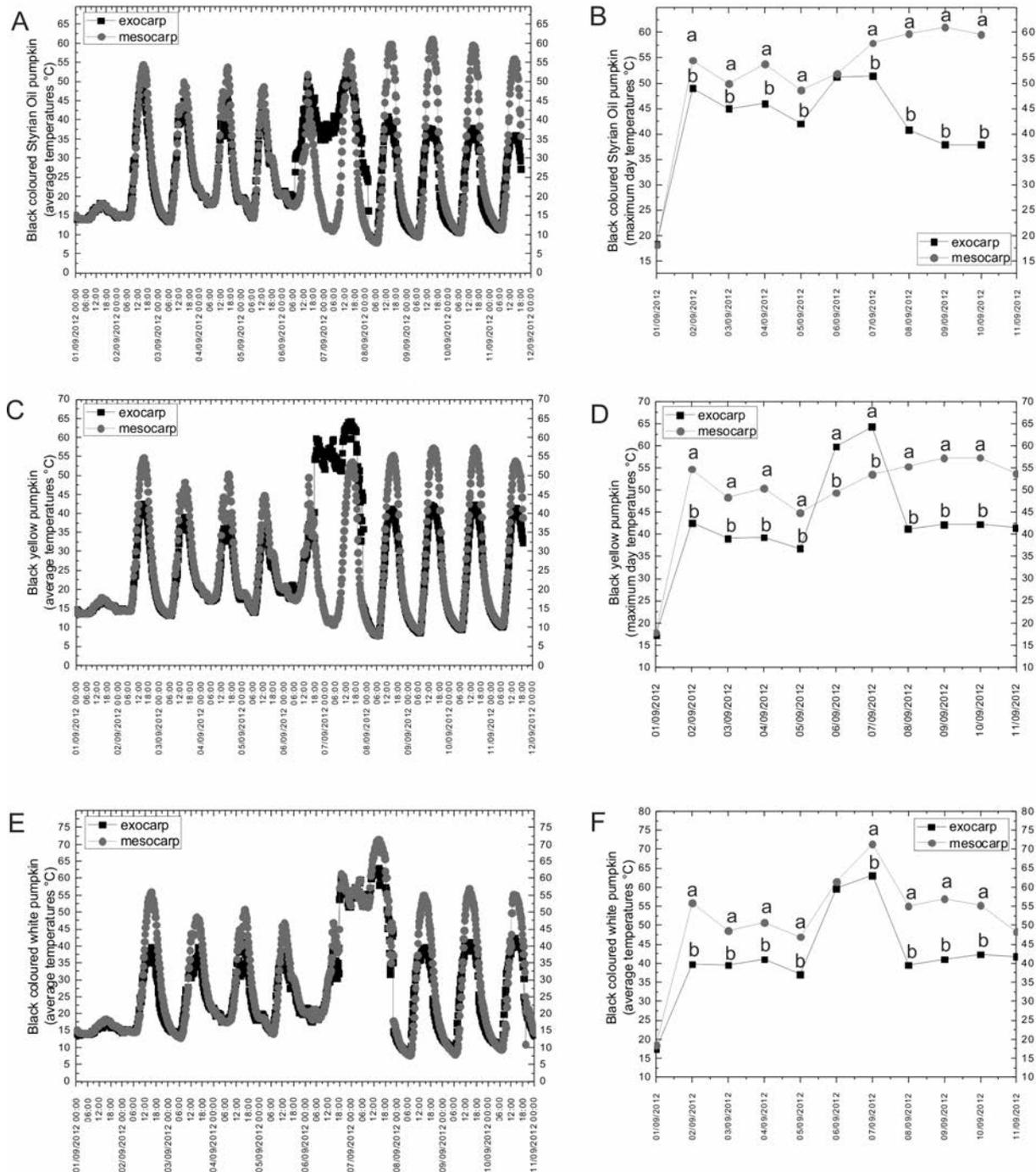


Fig. 6 - Average and maximum day temperatures measured in exocarp and mesocarp of black coloured detached pumpkins measured in the period between 1.-11. September 2012: A, B) Styrian oil pumpkin (O). C, D) Genotype with yellow fruits involving *C. argyrosperma*, *C. moschata* and *C. pepo* (A/Mo × O/A). E, F) Genotype with white fruits obtained from the cross *C. pepo* (non-lignified seed coat, oil type) × *C. argyrosperma* (O/A).

attached pumpkins the exocarp of the black coloured 'yellow' pumpkin heated up to 64°C, whereas the endocarp up to 48°C during the July-August period (Fig. 5C, D). Compared to the Styrian oil pumpkin the temperatures were higher. A rapid increase of temperature was determined in the late morning hours until noon. Later, an abrupt decrease of temperature followed and maintained at around 38°C between 3 and 6 p.m. After that, during the night hours, the temperature dropped below 20°C (Fig. 5C). Similarly, in September, during the early afternoon hours of the hottest day (7th September), the maximum day temperatures on exocarp reached 64°C, whereas in mesocarp the temperatures reached 57°C. On later dates the daily maximum temperatures of exocarp constantly reached 42°C, whereas mesocarp was heated up to 57°C (Fig. 6 C, D).

The 'white fruit' genotype (O/A) was characterised by the highest mesocarp temperatures during the hottest days of the 2th period when compared to O and A/Mo × O/A progenies. During the first five days, the maximum exocarp temperatures were around 40°C, whereas the mesocarp reached maximum day temperatures between 48°C and 55°C. During the following two days, the exocarp temperatures reached 60°C and 63°C, respectively, whereas the mesocarp 62°C and 71°C. Later, the exocarp maximum temperature was comparable to that of the black coloured 'yellow' pumpkin, reaching 42°C in the early afternoon hours, whereas the mesocarp was heated up to approx. 55°C (Fig. 6 E, F).

Summarising the results of temperature measurements within the black coloured pumpkins, we can conclude that for the attached pumpkins, the 'yellow' genotype was heated up more than the Styrian oil pumpkin. By analysing the results of detached pumpkins during the first days of measurements, the highest temperatures of exo- and mesocarp were determined in the Styrian oil pumpkin, whereas the maximum day temperatures of both 'yellow' and 'white' genotypes did not differ significantly and were about 5°C lower than those of the Styrian oil pumpkin. However, on the hottest days (6th and 7th September), the temperatures of exocarp within 'yellow' and 'white' genotypes were 8°C higher than those of the Styrian oil pumpkin reaching 64°C. Consequently, when compared to the Styrian oil pumpkin, higher temperatures were also determined in fruit tissues of both studied interspecific hybrids.

During the last two centuries, pumpkins have been selected for their softer pericarp in order to ease the laborious and time-consuming hand-har-

vesting of seeds. However, the resulting pumpkins' genotypes became more susceptible not only to diseases but also to various types of abiotic stress such as heat stress and drought. In order to create a variety with harder pericarp and also better abscission of the fruit peduncle, those pumpkins with lighter exocarp were crossed with the wild species *C. okechobeensis* (Fig. 1 G, H), which is characterised by hard pericarp, good abscission of the peduncle and resistance to viruses. The selected hybrids were histologically evaluated and compared to the Styrian oil pumpkin and O/A hybrids with lighter pericarps. The Styrian oil pumpkin (O) was characterised by its thick cuticle, a 15-cell layer of chlorenchyma cells and a 2-3 cell layer of more or less isodiametric lignified cells between the chlorenchyma and parenchyma of the mesocarp (Fig. 7 A, B). Both the 'yellow' (A/Mo × O/A) as well as the 'white' (O/A) genotypes were characterised by a similar 2-3 cell layer of thick lignified cells although the cell walls of this layer as well as the cuticle were thicker (Fig. 7 C, F). Crosses of O/A genotypes with *C. okechobeensis* (Oke × O/A) became characterised by a thicker sclerenchymatic layer of 4-5 oblong cells in a radial direction, which were approx. 100 µm long (Fig. 7 G, H). This progeny represents a good material for the future breeding for increased heat tolerance. Furthermore, *C. okechobeensis* has been recognised as promising for interspecific hybridization due to its central position in the genus *Cucurbita* (Gong *et al.*, 2013).

We may conclude that the genetic breeding of oil pumpkins for heat tolerance is still in its infancy stage and warrants more attention than it has been given in the past. Considerable information is presently available regarding the physiological and metabolic aspects of plant heat-stress tolerance (Ara *et al.*, 2013 a, b, 2015). Furthermore, attempts have been made to include molecular marker technology for genetic characterization and/or development of plants with improved heat tolerance. Gong *et al.* (2013) analysed SSR polymorphisms on a large collection of *Cucurbita* materials in order to obtain an improved insight into the relationships amongst most of the species of the genus. Wild species *C. foetidissima* has been identified as resistant to numerous pathogens and pests but is most distant to other *Cucurbita* species. *Cucurbita pepo* and *C. ficifolia* were the most outlying of the mesophytic species. The clusters of the six remaining species form three pairs, *C. maxima* with *C. ecuadorensis*, *C. okechobeensis* with *C. lundelliana*, and *C. moschata* with *C. argyrosperma*. Due to the genetic distances

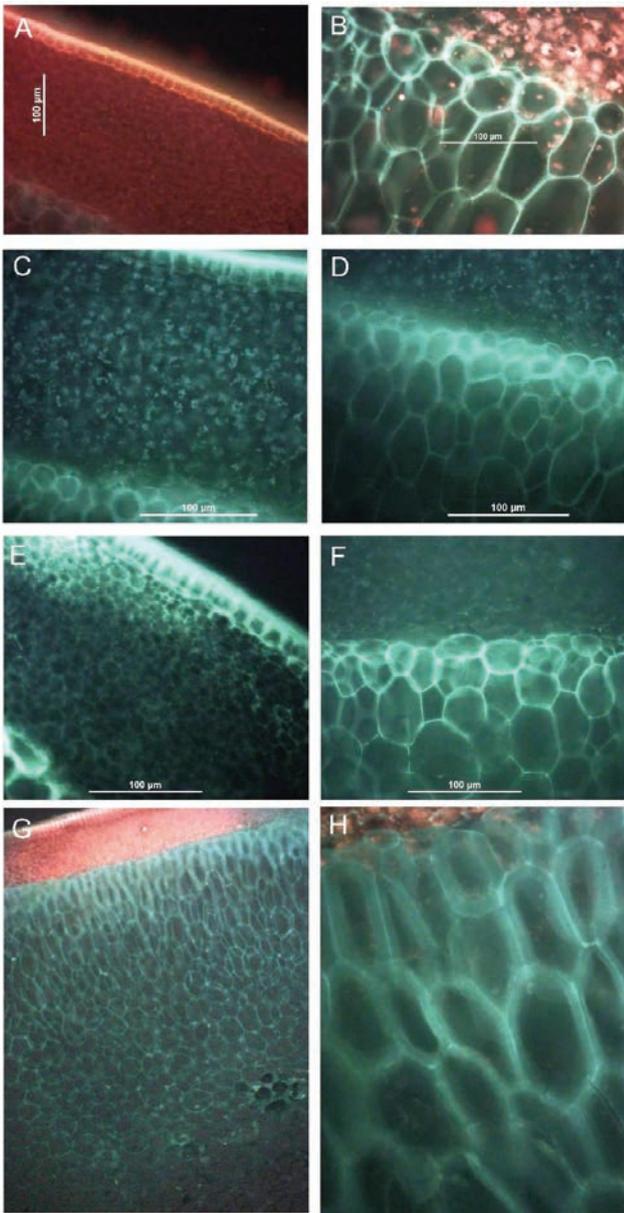


Fig. 7 - Histochemical analyses of perikarp. A, B) Styrian oil pumpkin (O) characterised by its thick cuticle, a 15-cell layer of chlorenchyma cells and a 1-2 cell layer of isodiametric cells and one layer of oblong cells with thick cell walls. C, D) Genotype with yellow fruits (A/Mo x O/A), with two layers of isodiametric cells with lignified cell walls. E, F) Genotype with white fruits (O/A) and three layer of lignified cells below the chlorenchyma. G, H) A three-species hybrid involving *C. pepo* (non-lignified seed coat, oil type), *C. argyrosperma* and *C. okechobeensis* (Oke x O/A) exhibiting 4-5 cell layers of oblong sklerenchymatic cells below the chlorenchyma.

amongst the *Cucurbita* species, various breeding strategies and biotechnological approaches have been employed (Merrick, 1995; Lebeda *et al.*, 2007; Ortiz-Alamillo *et al.*, 2007; Lelley *et al.*, 2009; Karaağaç and Balkaya, 2013) but the success in introgressing desirable traits from one species to another have been limited. *Cucurbita pepo* excels in plant

earliness and productivity but lacks genetic resources for disease resistance. *Cucurbita moschata*, on the other hand, carries resistance to various pathogens and is adapted to humid tropics but lacks earliness and productivity (Lebeda *et al.*, 2007; Lelley *et al.*, 2009; Karaağaç and Balkaya, 2013). Relatively high successes and fertilities have been observed for the cross-combination of *C. argyrosperma* and *C. moschata* (Montes-Hernandez and Eguiarte, 2002; Ortiz-Alamillo *et al.*, 2007) and *C. maxima* x *C. moschata* (Ara *et al.*, 2013 a, 2015).

However, despite all the complexity of heat tolerance and difficulties encountered during the genetic transfer of tolerance, few heat-tolerant inbred lines and hybrid cultivars with commercial acceptability have been developed and released (Montes-Hernandez and Eguiarte, 2002; Ortiz-Alamillo *et al.*, 2007; Ara *et al.*, 2013 a, b, 2015).

4. Conclusions

The presented study suggests that the colour of exocarp is probably one of the key parameters of tolerance to high temperatures. Darker colours are generally associated with higher fruit temperatures. In general, for attached pumpkin fruits higher temperatures were measured on exocarp, followed by meso- and endocarp. However, for the detached fruits on extremely hot days in September, the temperatures within mesocarp increased above those of exocarp. It is well known that if transpiration is interrupted by stomatal closure due to water stress and heat stress, a major cooling mechanism is not functioning. This was the reason of rising the internal fruit temperatures in case of detached fruits.

One could expect that the genotypes with whitish exocarp (i.e., O/A progenies derived from the cross Styrian oil pumpkin x *C. argyrosperma*) would be the most tolerant to heat stress. The experiment on detached fruits, however, suggest that the most tolerant is the A/Mo x O/A progeny derived from the cross involving the Styrian oil pumpkin, *C. argyrosperma* and *C. moschata*, which is characterized by yellow exocarp. One of the reasons for this could be the introgression of desirable traits of *C. moschata*, which is more adapted to high temperatures. This parental species was brought from the Island of Espiritu Santo, Vanuatu (tropical Pacific) and was obviously more tolerant to heat stress than the other two involved pumpkin species. The second important parameter appears to be the histological structure of

the pericarp. The genetic improvement of this complex trait should consider both parameters. Nevertheless, to accelerate such progresses, major areas of emphasis in the future should be: (1) development of accurate screening procedures at each stage of plant development; (2) identification and characterization of additional genetic resources associated with heat tolerance; (3) discerning the genetic inheritance of heat tolerance; (4) development and efficient screening of large breeding populations to facilitate transfer of genes for heat tolerance to commercial cultivars.

Acknowledgements

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The impact of fruit temperature dynamics on heat stress tolerance of selected oil pumpkin genotypes

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SUPPLEMENTARY MATERIAL

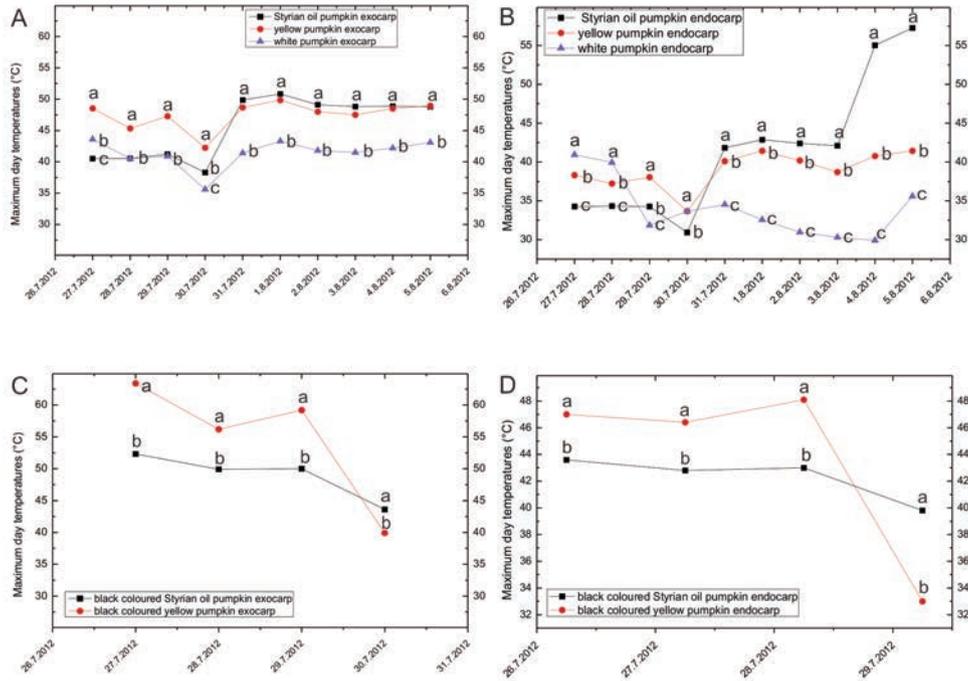


Fig. S1 - Comparison of the maximum day temperatures measured in the period between 27 July - 6 August 2012 in: A) Exocarp of Styrian oil pumpkin, yellow and white genotype; B) Endocarp of Styrian oil pumpkin, yellow and white genotype; C) Exocarp of black coloured Styrian oil pumpkin and yellow genotype; D) Endocarp of black coloured Styrian oil pumpkin and yellow genotype.

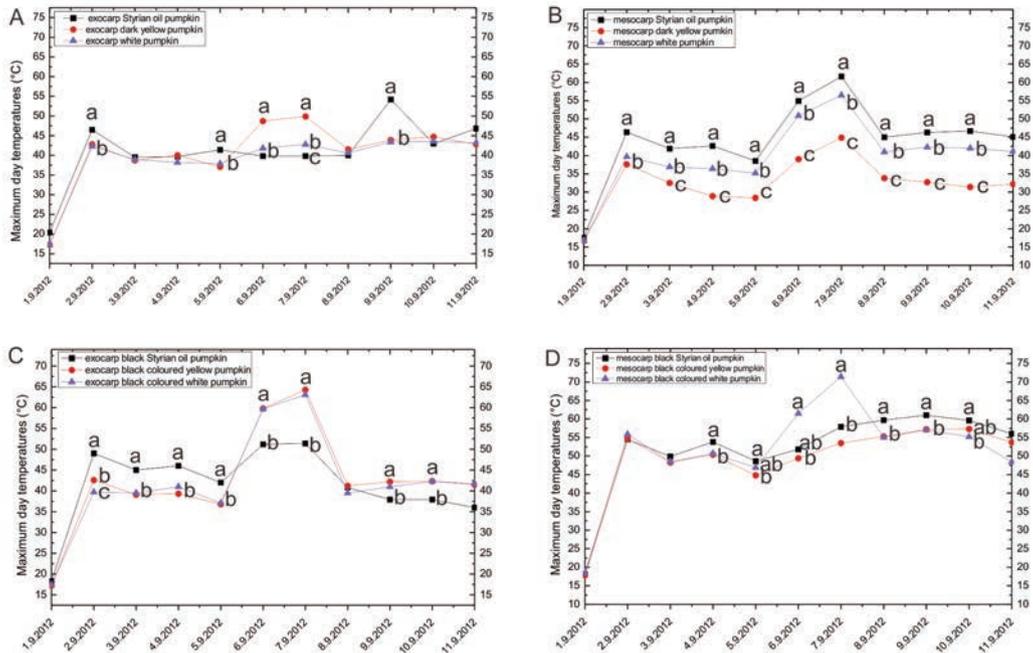


Fig. S2 - Comparison of the maximum day temperatures measured in the period between 1-11 September 2012 in: A) Exocarp of Styrian oil pumpkin, dark yellow and white genotype; B) Mesocarp of Styrian oil pumpkin, yellow and white genotype; C) Exocarp of black coloured Styrian oil pumpkin, yellow and yellow genotype; D) Mesocarp of black coloured Styrian oil pumpkin, yellow and yellow genotype.