Doctoral Dissertation (Shinshu University)

Effect of environmental condition on capsaicinoid, sugar content and expression of capsaicinoid biosynthesis genes in chili pepper (*Capsicum* spp.)

“トウガラシ（*Capsicum* spp.）のカプサイシノイドおよび糖含量ならびにカプサイシノイド合成関連遺伝子の発現に及ぼす環境の影響”

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Table of Contents

Chapter I

General Introduction

Chili has been reported to be in use since the ancient stages of agriculture and is one of the oldest domesticated crops in the Western hemisphere (Pandit et al., 2020). After tomato and potato, chili is the third most important crop in the family Solanaceae (Naz, 2006). *Capsicum annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescen* are the five domesticated species of the genus *Capsicum*, which contains more than 30 species (Moscone et al., 2007). *C. annuum* is the most popularly used chili as a vegetable as well as a food additive. Based on archeological, paleoclimatic, mid-holocene, linguistic, and genetic data, Mexico was probably the center of origin and domestication of *C. annuum* (Kraft et al., 2014).

Capsaicinoids are the active ingredients responsible for the pungency or hotness of the chili fruits. Capsaicin and dihydrocapsaicin are major capsaicinoids, accounting for more than 90% of the total capsaicinoid content in the majority of *Capsicum* spp. (Nunez-Palenius and Ochoa-Alejo, 2005). Phenylpropanoid pathways and branched- chain fatty acid pathways are involved in the synthesis of capsaicinoid (Aza-Gonzalez et al., 2011). Vanillylamine from the phenylpropanoid pathway and 8-methyl-6-nonenoyl-CoA from the branched-chain fatty acid pathway, condense together to form capsaicinoid (Ogawa et al., 2015).

In tomato cultivation, the quality of fruit flavor is determined by sugar (fructose and glucose), organic acid content (primarily citric, malic, and total acidity), and volatile compound composition (Mikkelsen, 2005). However, in chili peppers, the capsaicinoids, sugar, and Brix (total solid content) are the important taste components for the food industry.

Capsaicinoid accumulation depends on genetic factors of the cultivar as well as environmental factors such as water availability, salinity, light intensity, and fertilizer availability (Buczkowska et al., 2013). Drought (Rathnayaka et al., 2021c), salinity (Rathnayaka et al., 2021b), N (nitrogen) fertilizer stress (Medina-Lara et al., 2008) and parthenocarpy (Kondo et al., 2020; 2021) are the environmental factors that increase the capsaicinoid content. It has been also reported that capsaicinoid biosynthesis is affected by the age, size, and developmental stage of the fruit (Estrada et al., 1997; Zewdie and Bosland, 2000). Light is the environmental factor which has the highest effect on the fruit sugar concentration in tomatoes (Mikkelsen, 2005). Changes in the pungency and sweetness of chili peppers due to the environmental conditions has been a big issue for the food industry.

Plants can get lots of elements through the air and produce glucose and other substances with the aid of sunlight. However, N, phosphorus (P) and potassium (K) are plant elements which cannot be absorbed through air or cannot be produced through photosynthesis (https://www.fertilizer-machine.net). According to Medina-Lara et al. (2008), N fertilizer significantly increased plant growth and capsaicinoid content and K fertilizer did not show any positive effect with growth or productivity of chili fruits.

Lots of studies have been done to determine the effects of organic N, K and organic fertilizer on chili capsaicinoid content, but few studies on the effects of P fertilizer are reported. Japanese agricultural lands have a high level of P fertilizer because of excessive P fertilizer application (Mishima et al., 2010).

The capsaicinoid biosynthesis pathway has many genes which have been discovered so far (Qin et al., 2014). Capsaicinoid biosynthetic genes are expressed preferentially as a response to various factors such as genotype, developmental stage, and environmental conditions. However, the expression levels of most of these genes under different regulation conditions are yet to be studied (Aza-Gonza´lez et al., 2011). Excess P also could be a reason for variable expression levels of the genes in the capsaicinoid biosynthesis pathway.

Therefore, the two of the objectives of this study were to determine the relationship between P fertilizer with capsaicinoid, sugar, total sugar content, and Brix percentage, and also, to determine the expression level of the capsaicinoid biosynthesis gene with excess P fertilizer application. Experiments described in Chapter II and III have been conducted to investigate the above-mentioned objectives, respectively.

Another major environmental factor that affects capsaicinoids, sugar, Brix percentage, and glutamic acid is temperature. With the increasing temperature due to global warming, amount of these taste components produced in chili fruits vary and it has been an issue with the quality of chili. However, the available research information on this topic is extremely limited. Therefore, there is a need for research experiments to test the effect of temperature on chili. Therefore, the other objective of this study is to investigate the effect of temperature stress on the abovementioned taste components, as described in experiments conducted in Chapter IV.

Chapter II

Effect of Soil Phosphorus Levels on Capsaicinoid and Sugar Content in Chili Pepper (*Capsicum* spp.)

Abstract

The capsaicinoid and sugar content are important for the flavor of foods, and the concentrations of these compounds vary with changing environmental factors. The present multi-year experiment was conducted to determine the effect of soil phosphorous on capsaicinoid levels and sugar content in the fruit of three *Capsicum annuum* varieties (‘Takanotsume’, ‘Sapporo Oonaga Namban’ and ‘Shishito’) and one *C. chinense* variety (‘Habanero’). Experiments were conducted under the greenhouses at Shinshu University (Minamiminowa, Nagano). In 2018 and 2020, different levels of phosphorus fertilizer were applied and the flavor components, including capsaicinoid, of the fruits were measured. The capsaicinoid content increased from 100 to 200 $g{\cdot}m^{-2}$ and then decreased at the 300 $g{\cdot}m^{-2}$ phosphorus fertilizer applied in ‘Takanotsume’, ‘Sapporo Oonaga Namban’ and ‘Habanero’. These results reveals that the capsaicinoid content increases with the increment of phosphorus fertilizer and tended to decrease with excess phosphorus application. There was a tendency for the total sugar and glucose content to increase slightly when the plants were grown in soil with high phosphorus (300 $g{\cdot}m^{-2}$ and 600 $g{\cdot}m^{-2}$).

Introduction

Chili pepper fruits are produced by plants in the genus *Capsicum*, a part of the Solanaceae family. This plant genus produces capsaicinoids (Gurung et al., 2011). *Capsicum annuum* is one of five commonly cultivated domesticated species of the *Capsicum* genus (Kraft et al., 2014) and the hottest chilies are produced by *Capsicum chinense* (Bosland and Baral, 2007). The pungency, hotness and burning sensation caused by chili are due to the active ingredient capsaicinoid (Buczkowska et al., 2013). The pungent fruit is grown in a wide area, from the tropics to temperate zones, and is mainly used as a spice and pungent vegetable. Capsaicin and dihydrocapsaicin are the two major capsaicinoids responsible for pungency and are synthesized by plants carrying the *Pun1* gene through a combination of the phenylpropanoid and branched-chain fatty acid pathways (Aza-Gonzalez et al., 2011). The amount of capsaicinoid in hot pepper is dependent on genetic traits, and on the environmental and weather conditions during fruit set and growth, as well as conditions during fertilization (Buczkowska et al., 2013; Topuz and Ozdemir, 2007) such as drought stress, salinity stress (Rathnayaka et al., 2021c;2021b) and parthenocarpy (Kondo et al., 2020; 2021).

Sweetness is an important basic taste in chili pepper and is due to glucose and fructose. In tomatoes, the accumulation of sugar (glucose and fructose) and organic acid depends on environmental conditions such as temperature, solar radiation, water conditions, fertilization, and the plant's growth phase (Rosales et al., 2011). According to Mikkelsen (2005), high sugar and acid levels in tomatoes result in a favorable taste. In contrast, changing the pungency and sweetness of peppers remains an unaddressed problem in the food industry.

Several experiments have investigated the effect of fertilizer nitrogen (N), potassium (K) and organic compounds on capsaicinoid content. For example, Iwai et al. (1979) reported that N fertilizer significantly increased plant growth and fruit yield while maintaining high levels of capsaicin, whereas K levels did not affect the pungency of peppers (Charles and Dennis, 1996). When the organic fertilizer was added together with mineral fertilizers, five-time higher percentage of capsaicin was reported than that obtained with the mineral fertilizer treatment (Pampuro et al., 2017). Even low levels of fertilizer often enhance yields dramatically, although excessive fertilizer application is expensive and can negatively affect crop uptake of nutrients (Epstein and Bloom, 2005).

Phosphorus (P) is the second most essential soil nutrient element after N (Anonymous, 2002). Plants require P to synthesize adenosine triphosphate (ATP), sugars, and nucleic acids (McCauley et al., 2011). Japanese agricultural soil is typically enriched in P due to long-term excessive P fertilizer application (Altansuvd et al., 2014). There are various reports on the relationship between the P content of soil and capsaicinoid levels in peppers, but the relationship is unclear, since the capsaicinoid content also reportedly increases as the P content increases (Bajaj, 1979) or decreases (Sega, 1972). The effects of glucose, total sugar content and Brix parentage on capsaicinoid levels is also unclear.

Kitamura et al. (2009) conducted pot cultivation experiments with soil from two farmers' fields in Kinasa, Nagano city, and two research fields at Shinshu University (Minamiminowa and Minamimaki) in Nagano prefecture using the pungent chili pepper variety 'Takanotsume' and strain 'ST-2' in 2007. The results of this pot cultivation experiment using four field soils showed significant and strong negative correlations between the residual P level in the soils and the level of capsaicinoid in the fruits (Fig. 1), and significant positive correlations between the residual N levels and the capsaicinoid content, but no relationship with K levels (Table 1).

There was a high correlation between P content in the soil and the capsaicinoid content in the fruit in the above pot cultivation experiment and they have investigated changes in capsaicinoid content in fruit by varying the P content in the soil in the main research project, as follows.

Six P fertilizer treatments (0, 15, 30, 45, 60, and 75 g·m^{-2}) were applied to the soil and the plants were grown in the research field outdoors (Kitamura et al., 2009). Kawaguchi, (personal contacts) done same experiment using six P fertilizer treatments 2010 (0, 15, 30, 60, 120, 180 and 240 g·m^{-2}) and 2011(0, 60, 120 and 240 g·m^{-2}).

The capsaicinoid content of ‘Takanotsume’ from experiments Kitamura et al. (2009) in 2008 and Kawaguchi, (personal contacts) in 2010 and 2011 were shown in Figure 2. In Kitamura et al. (2009) experiment, conducted in 2008, all ‘Takanotsume’ fruits grown with P applied to the soil had significantly higher capsaicinoid content than those grown without added P.

In Kawaguchi, (personal contacts) experiment, the capsaicinoid content of ‘Takanotsume’ significantly increased upon the application of up to 120 g·m^{-2} P, and remained constant at 180 and 240 g·m^{-2} P (Fig. 2). The same experiment conducted in 2011 showed a similar tendency but no significant difference was observed between P fertilizer treatment (0, 60, 120 and 240 g·m^{-2}) and capsaicinoid content.

Therefore, with the assumption of the amount of N, P and K remaining in the utilized soil would affect the capsaicinoid content and the sugar content of chili pepper fruits, the present study was conducted to investigate the relationship between P levels in fertilized soil and capsaicinoid content, as well as the effect of P levels on the content of glucose, total sugar and Brix percentage.

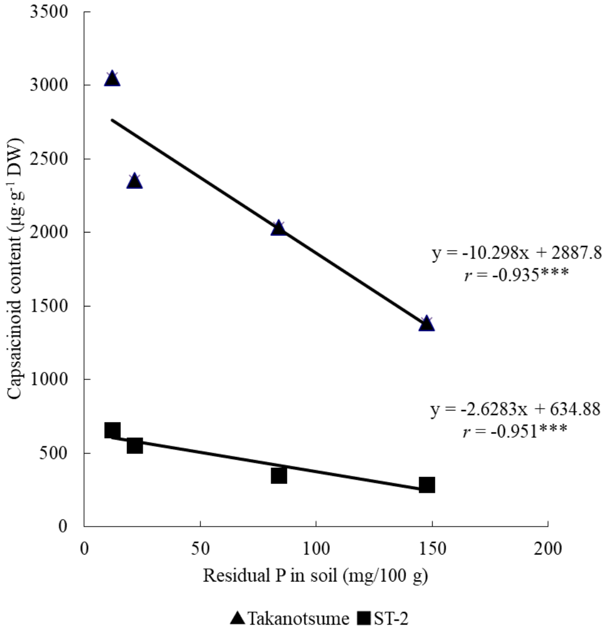


Fig. 1. Correlation between the amount of soluble phosphate remaining in the soil in four different farm fields in Nagano prefecture and the capsaicinoid content in the fruit of two *Capsicum annuum* varieties ('Takanotsume' and 'ST-2') grown in each farm field in 2007. *** indicates significance at the 0.001 level. (1mg/100g ≈ 5.88 $g \cdot m^{-2}$)

Table 1. Correlation coefficients between the amount of fertilizer component remaining in soil collected from four different fields in Nagano prefecture and the capsaicinoid content in chili pepper fruits grown in pots using each soil in 2007. * and *** indicate significance at the 0.05 and 0.001 levels, respectively.

Chili Varieties	Fertilizer component		
	N	P	K
Takanotsume	0.740^{*}	-0.935^{***}	-0.387
ST-2	0.837^{***}	-0.951^{***}	-0.655

(Kitamura et al., 2009)

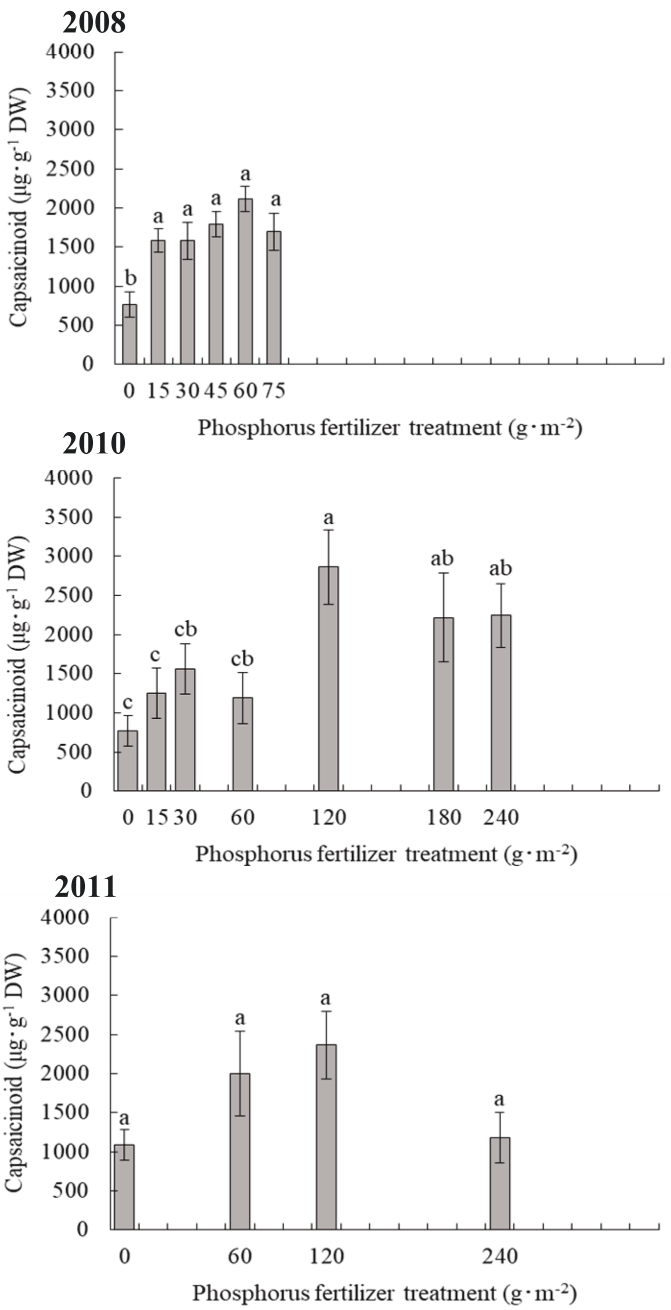


Fig. 2. Capsaicinoid content [μg·g^{-1} dry weight (DW)] for 'Takanotsume' grown in soil with different levels of P (phosphorous) in 2008, 2010 and 2011. Different lower-case letters (a, b and c) indicate significant differences between treatments (Tukey's pairwise test, $p<0.05$). Error bars indicate the standard error.

(Kawaguchi, personal contacts)

Materials and methods

The main research project comprised two experiments conducted from 2018 to 2020 in the research field of the AFC, Faculty of Agriculture, Shinshu University. Three *C. annuum* varieties (‘Takanotsume’, ‘Sapporo Oonaga Namban’, and ‘Shishito’) and one *C. chinense* (‘Habanero’) variety were used at different stages of the research. ‘Sapporo Oonaga Namban’ (hereinafter referred to as ‘Sapporo’, Tsurushin Seeds, Matsumoto, Japan) is a local chili pepper variety from Hokkaido, and has larger fruits and is less pungent than ‘Takanotsume’ fruit. ‘Shishito’ (Sakata Seeds, Kanagawa, Japan) is the most common vegetable pepper variety grown in Japan, whereas ‘Habanero’ (Tsurushin Seeds, Matsumoto) is a chili pepper originating from a local Mexican variety and is one of the most pungent chili pepper varieties in the world.

Common cultivation practices followed throughout two experiments

Seedlings approximately 10 cm high grown in a greenhouse were transplanted into plastic pots (18 cm in diameter, 2.2 L) filled with non-fertile soil (Shinano-Baiyodo Corporation, Nagano, Japan) which does not having any fertilizer in soil, in Experiment 1. Both Experiments, Ammonium sulfate [$(NH_4)_2SO_4$, available N: 21%], potassium chloride (KCl) (available K: 60%), and magnesium lime ($MgCO_3.CaCO_3$, available Mg: 15%) were applied to the soil in the pots at the recommended levels of 30, 24 and 15 $g{\cdot}m^{-2}$, respectively by Nagano prefecture for cultivation of sweet peppers. The commercial product Multi Phosphate (National Federation of Agricultural Cooperative Associations, Tokyo, Japan, available P: 35%) was used as a P fertilizer and was added as described below. In Experiment 2, the same size plants were transplanted into clay pots (18 cm in diameter, 3.5 L) filled with commercial potting media (Nae-Ichiban; Sumitomo Forestry

Landscaping Co., Ltd, Tokyo, Japan). The first set of flower buds was removed after transplanting. Water was applied as required, taking into account the daily temperature and weather conditions. If the temperature was above 30 °C, 130 mL of water was added to the soil 3 times a day. On rainy days or if the day temperature was below 30 °C, 130 mL of water was applied twice a day. Other crop management practices followed standard recommendations for growing peppers in Japan.

After fruits appeared, each plant was supported with a pole and the fruit was harvested when fully red because the capsaicinoid content increases during fruit development and decreases after the pericarp turns red (Minami et al., 1998). The collected harvest was stored in a deep freezer (-80 ℃) until analysis.

Five plants were used for each treatment in both experiments, with 1-3 fruits from each plant harvested and used as samples for capsaicinoid analyses. Fruits bulks were collected from plants subjected to each treatment, divided into three similar samples, and used for taste component analyses in experiments 1 and 2. The capsaicinoid content of the fruit was measured in experiment 1 and the glucose and total sugar contents and Brix percentage were measured in experiments 1 and 2.

Experiment 1 for capsaicinoids and taste components (conducted in 2018)

Experiment 1 was conducted from 2nd May to 9th October, 2018, using ‘Takanotsume’, ‘Sapporo’ and ‘Habanero’ varieties and four P fertilizer treatments (0, 100, 200 and 300 $g{\cdot}m^{-2}$). The experiment was conducted in a greenhouse with 50% shading, and the fruits were harvested twice, in the summer (29th of July to 2nd of September) and in the autumn (9th of September to 10th of October) in 2018. The maximum average temperature was 34.8 °C and the minimum average temperature was 17.5 °C.

Experiment 2 for taste components (conducted in 2020)

The experiment was conducted using three P fertilizer treatments (60, 300 and 600 g·m^{-2}) and three varieties ('Takanotsume', 'Sapporo' and 'Shishito') in 2020. Commercial potting medium usually contains 60 g·m^{-2} equivalent of P fertilizer and therefore 60 g·m^{-2} of commercial pot medium was used as the standard, and plots with excess P (300 g·m^{-2} and 600 g·m^{-2}) were prepared by adding P fertilizer. The experiment was conducted in a greenhouse with 50% shading; the maximum average temperature was 35 °C and the minimum average temperature was 20.5 °C.

Capsaicinoids analysis

Frozen (-80 °C) fruit samples were freeze-dried using a freeze-dryer (Islay FDU – 2000, Rikakikai Co., Ltd, Tokyo, Japan) for 24 hours, pulverized (YMB-400; Yamazen, Osaka, Japan), then 20 mL of methanol was added to the dried pepper powder and the sample was incubated at 40 °C for 1 hour (Natural Incubator, Compact NIB-10, Iwaki Glass Co., Ltd, Tokyo, Japan) to extract the capsaicinoid. The extraction sample was filtered through 125 mm filter paper 6 µm pore size filter paper (ADVANTEC®, Tokyo, Japan) and methanol was again added up to 20 mL.

Capsaicinoid was analyzed using high-performance liquid chromatography (HPLC) (YMC-Pack ODS-A, 50 × 3.0 mm, Shimadzu Corporation, Kyoto, Japan), with a column temperature of 40 °C, mobile phase of 65% methanol, a flow rate of 1 mL·min^{-1}, and monitoring at 280 nm.

Preparation of solution to analyze glucose and total sugar

Frozen chili fruit samples were ground, filtered using 125 mm 6 µm pore size filter paper filter paper (ADVANTEC®, Tokyo, Japan), then diluted 100 times, and glucose, total sugar and glutamic acid content were quantitatively analyzed using a portable spectrophotometer (RQ flex plus 10; Merck, Darmstadt, Germany).

Preparation of solution to analyze Brix

Extracts prepared as above were directly used to read the Brix value using a digital portable refractometer (Pen-J; ATAGO Co., Ltd, Tokyo, Japan).

Results

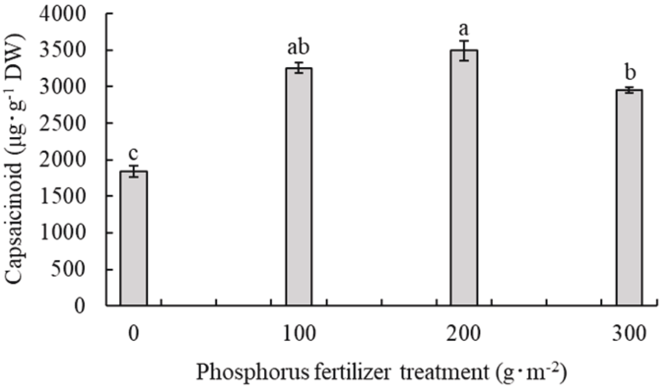
Capsaicinoid content

In Experiment 1, conducted in 2018, 'Takanotsume' fruits harvested in the summer showed the lowest capsaicinoid content at 0 g·m^{-2} P and significantly higher capsaicinoid content when grown in soil treated with 200 g·m^{-2} P fertilizer compared to 0 g·m^{-2} P and 300 g·m^{-2} P (Fig. 3). Autumn harvested 'Takanotsume' fruits showed a similar tendency, except the highest capsaicinoid content was in fruits grown in soil treated with 100 and 200 g·m^{-2} P fertilizer. 'Takanotsume' grown in 100 g·m^{-2} and 300 g·m^{-2} P fertilizer treatment showed significantly higher capsaicinoid content in autumn than the summer.

'Sapporo' fruits harvested in summer and grown in 0 g·m^{-2} P showed significantly lower capsaicinoid content than plants grown in soil treated with 100 g·m^{-2} P fertilizer (Fig. 4). There were no significant differences between 'Sapporo' fruits harvested in the summer and grown in soil treated with 100, 200 and 300 g·m^{-2} P. Autumn harvested 'Sapporo' fruits grown in soil treated with 0 and 300 g·m^{-2} P fertilizer showed significantly lower capsaicinoid content than fruits harvested from plants grown in soil treated with 100 and 200 g·m^{-2} P fertilizer. Fruits from the 100 g·m^{-2} P pots contained higher capsaicinoid levels than those from 200 g·m^{-2} P pots harvested in autumn. There were no significant differences in capsaicinoid content between summer and autumn in 'Sapporo' fruits harvested from plants grown at each fertilizer level.

'Habanero' fruits had much higher capsaicinoid content than the other two varieties (Fig. 5). Summer-harvested 'Habanero' fruits exhibited higher capsaicinoid content when grown in soil treated with 100, 200 and 300 g·m^{-2} P fertilizer than 0 g·m^{-2} P fertilizer, whereas those harvested in the autumn had lower capsaicinoid content if grown in soil treated with 0 or 300 g·m^{-2} P fertilizer compared to 100 and 200 g·m^{-2} P. For 'Habanero', only plants grown in 300 g·m^{-2} P fertilizer showed significantly different capsaicinoid content between summer and autumn.

2018 Summer



2018 Autumn

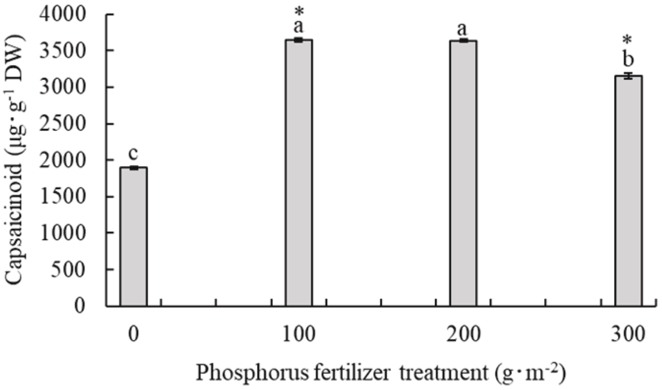
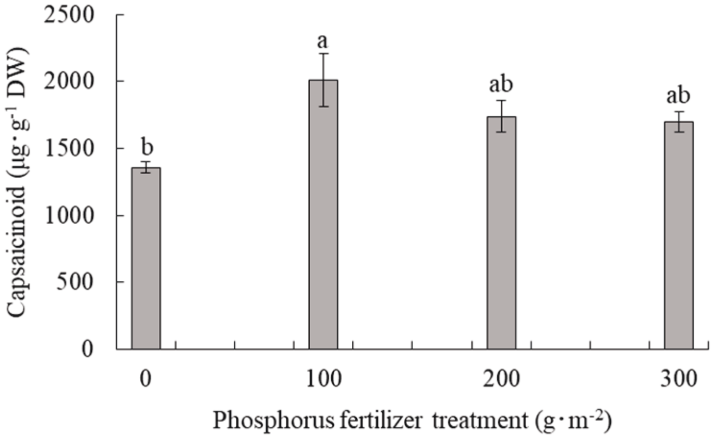


Fig. 3. Capsaicinoid content [μg·g^{-1} dry weight (DW)] for 'Takanotsume' chili peppers grown in soil with different levels of P (phosphorous) in 2018 summer and 2018 autumn. Different lower-case letters (a, b and c) indicate significant differences between treatments. * indicate significant differences in each P treatment between the summer and autumn (Tukey's pairwise test, $p<0.05$). Error bars indicate the standard error.

S, 2018 Summer



S, 2018 Autumn

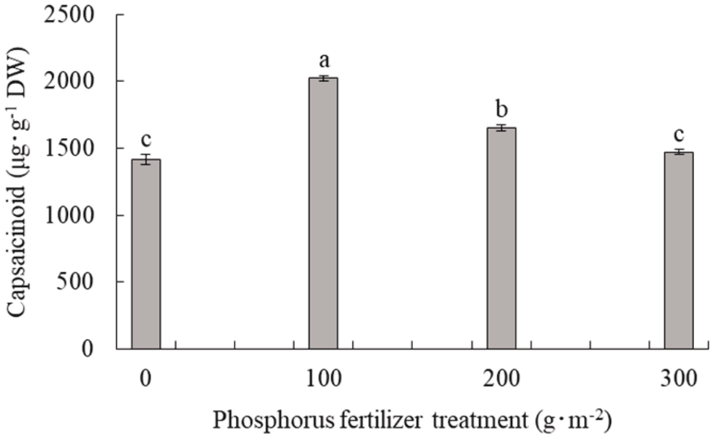
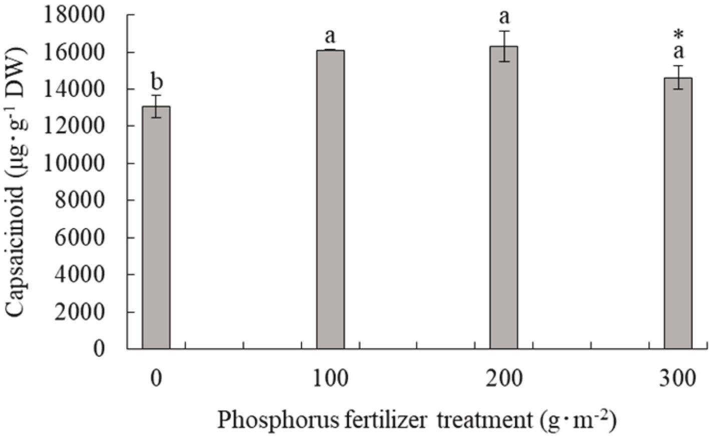


Fig. 4. Capsaicinoid content [$\mu g \cdot g^{-1}$ dry weight (DW)] in ‘Sapporo’ (S) chili peppers grown in soil with different levels of P in 2018 summer and autumn. Different lower-case letters (a, b and c) indicate significant differences between treatments for ‘Sapporo’. (Tukey’s pairwise test, $p<0.05$). Error bars indicate the standard error.

H, 2018 Summer



H, 2018 Autumn

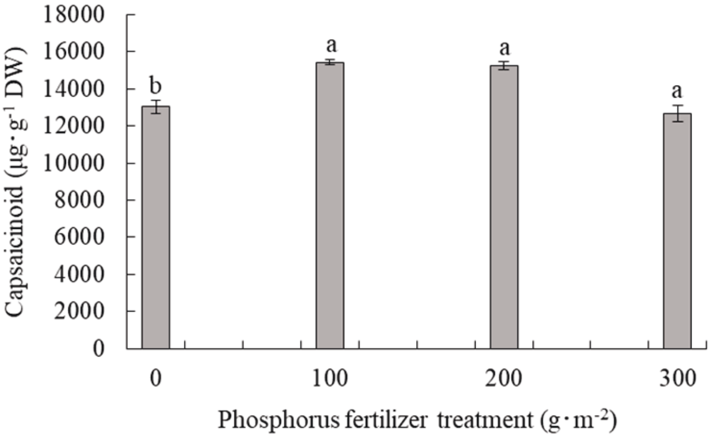


Fig. 5. Capsaicinoid content [$\mu g \cdot g^{-1}$ dry weight (DW)] in ‘Habanero’ (H) chili peppers grown in soil with different levels of P in 2018 summer and autumn. Different lower-case letters (a, b and c) indicate significant differences between treatments for ‘Habanero’. * indicate significant differences in each P treatment between the summer and autumn (Tukey’s pairwise test, $p<0.05$). Error bars indicate the standard error.

Glucose content

'Takanotsume' and 'Sapporo' plants were grown in 2018 in soil treated with 300 g·m^{-2} P fertilizer had significantly higher glucose content than those grown in 0, 100 and 200 g·m^{-2} P (Fig. 6), whereas the glucose content in 'Habanero' fruit increased with increasing P fertilizer content. There were significant differences of glucose content in 'Habanero', between 0 and 100 g·m^{-2} P, 100 and 300 g·m^{-2} P and 0 and 300 g·m^{-2} P fertilizer treatments. 'Sapporo' showed the highest glucose content at all P fertilizer levels compared to 'Takanotsume' and 'Habanero'. Experiment 2, conducted in 2020, tested a wider range of P application levels. The glucose content of 'Sapporo' and 'Takanotsume' significantly increased when grown in soil treated with 60 to 300 g·m^{-2} P, and remained unchanged at 300 to 600 g·m^{-2} P (Fig. 7), whereas 'Shishito' showed higher glucose only in the 600 g·m^{-2} P-treated soil compared to the 60 g·m^{-2} P soil.

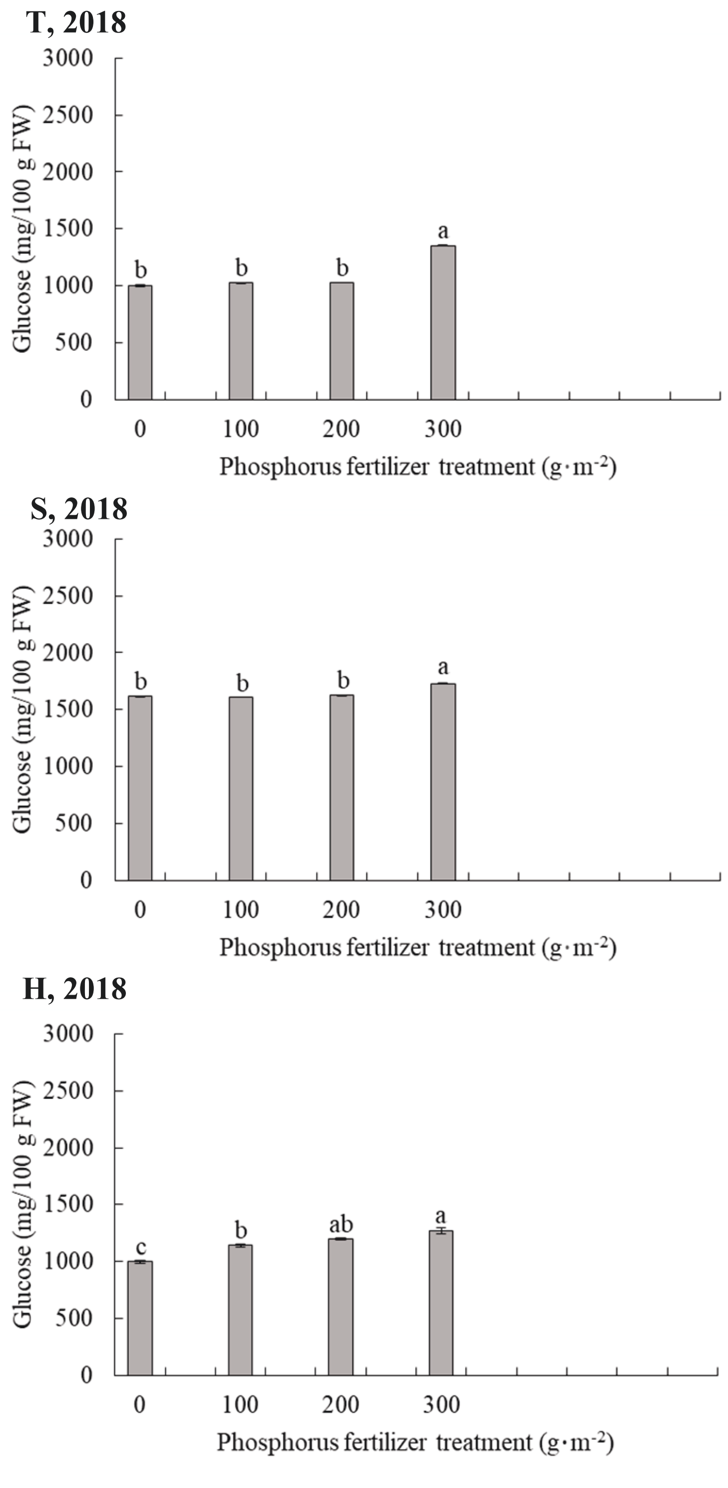


Fig. 6. Glucose content [mg/100 g fresh weight (FW)] of ‘Takanotsume’ (T), ‘Sapporo’ (S) and ‘Habanero’ (H) chili pepper fruits in 2018. Different lower-case letters (a, b and c) indicate significant differences between treatments (Tukey’s pairwise test, $p<0.05$) of ‘Takanotsume’ and ‘Sapporo’. Error bars indicate the standard error.

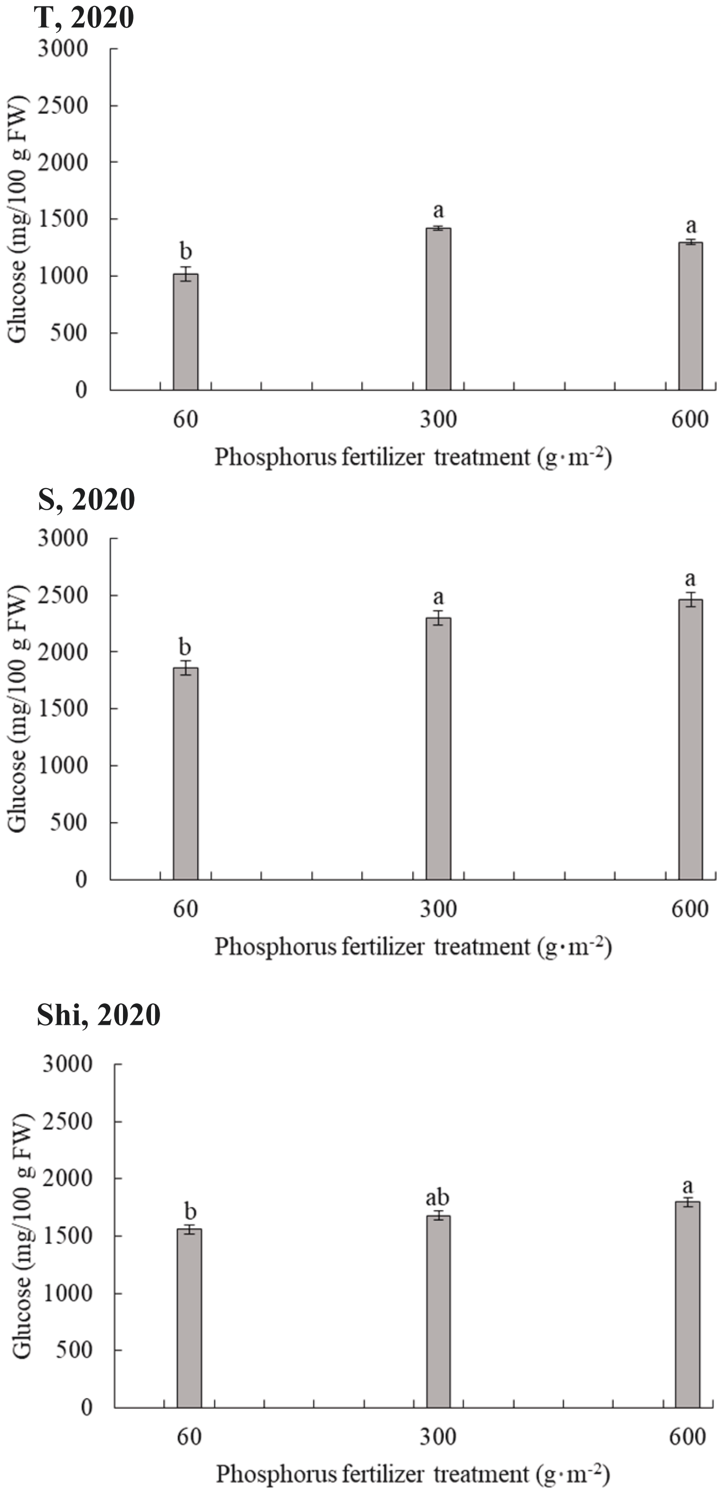


Fig. 7. Glucose content [mg/100 g fresh weight (FW)] of ‘Takanotsume’ (T), ‘Sapporo’ (S) and ‘Shishito’ (Shi) chili pepper fruits in 2020. Different lower-case letters (a and b) indicate significant differences between treatments (Tukey’s pairwise test, $p<0.05$) of ‘Takanotsume’, ‘Sapporo’, ‘Shishito’. Error bars indicate the standard error.

Total sugar content

'Takanotsume' and 'Sapporo' grown in soil treated with 300 g·m^{-2} P fertilizer showed higher fruit total sugar content compared to the other treatments in 2018 (Fig. 8). The total sugar content in 'Habanero' fruit increased with increasing phosphorus fertilizer content and there was a significant difference between 0 and 200 g·m^{-2} P and between 200 and 300 g·m^{-2} P. 'Sapporo' had a higher total sugar content at all P fertilizer levels than 'Takanotsume' and 'Habanero'.

In 2020, 'Takanotsume' grown under 60 to 600 g·m^{-2} P conditions showed significantly increased total sugar content. The total sugar content of 'Sapporo' was significantly higher when grown in soil treated with 300 and 600 g·m^{-2} P than in 60 g·m^{-2} P. Whereas no changes in total sugar content were found in 'Shishito' (Fig. 9).

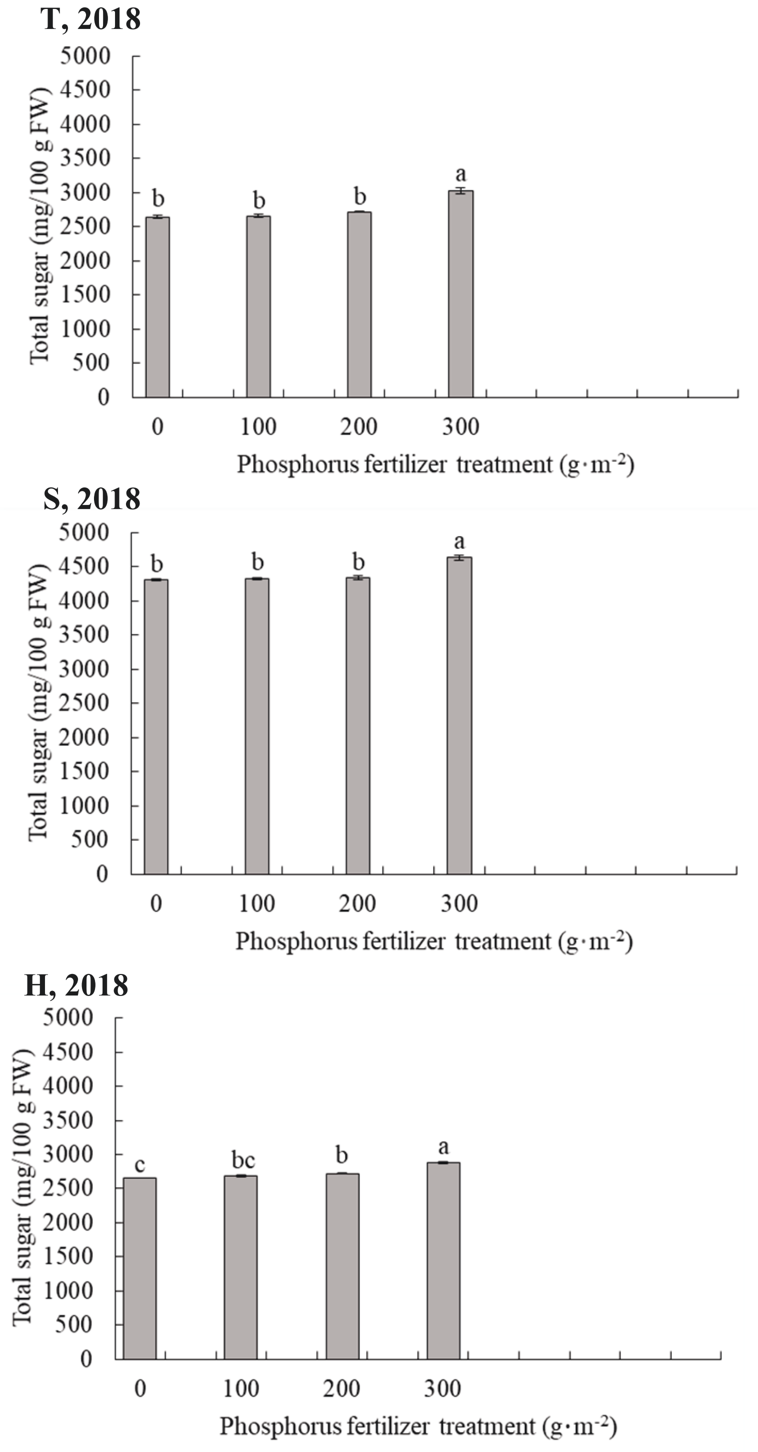


Fig. 8. Total sugar content [mg/100 g fresh weight (FW)] of ‘Takanotsume’ (T) ‘Sapporo’ (S) and ‘Habanero’ (H) chili pepper fruits in 2018. Different lower-case letters (a, b and c) indicate significant differences between treatments (Tukey’s pairwise test, $p<0.05$) of ‘Takanotsume’, ‘Sapporo’, ‘Habanero’. Error bars indicate the standard error.

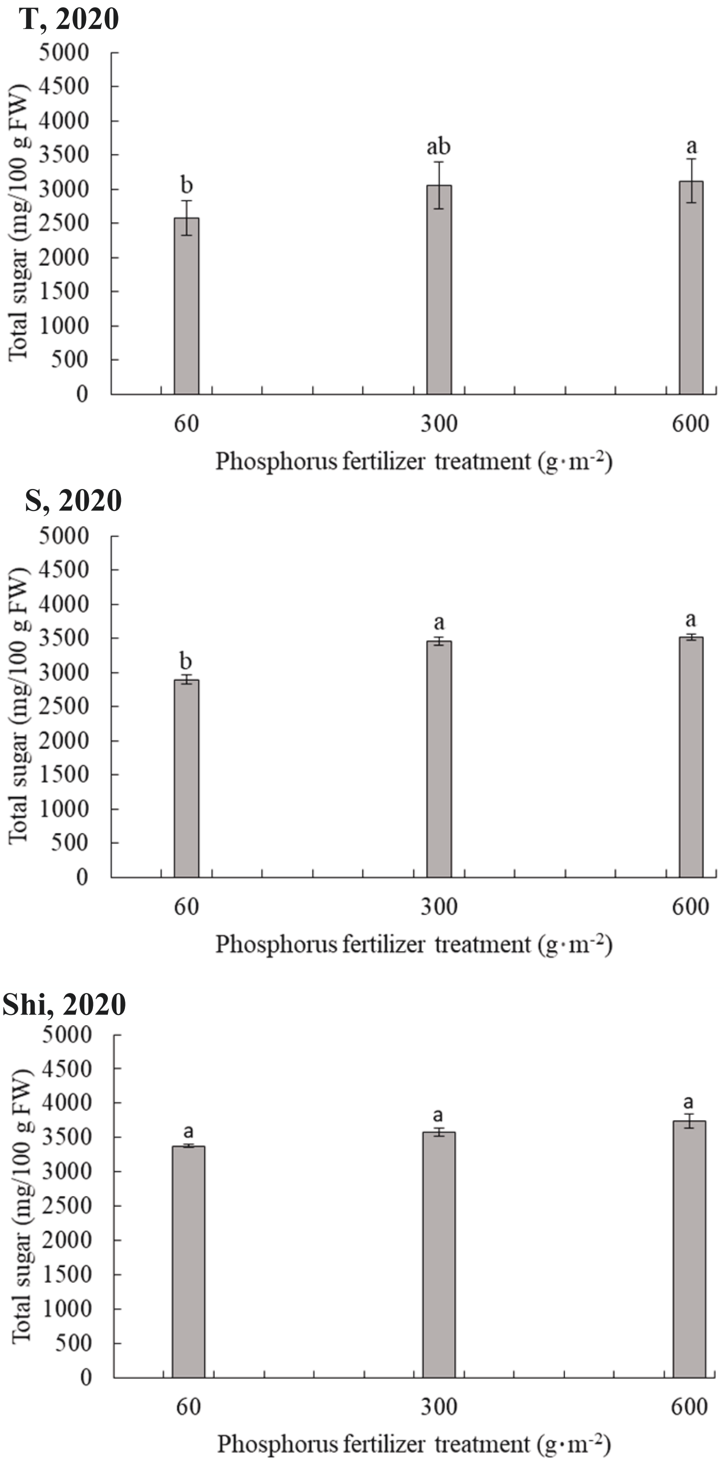


Fig. 9. Total sugar content [mg/100 g fresh weight (FW)] of ‘Takanotsume’ (T) ‘Sapporo’ (S) and ‘Shishito’ (Shi) chili pepper fruits in 2020. Different lower-case letters (a and b) indicate significant differences between treatments (Tukey’s pairwise test, $p<0.05$) of ‘Takanotsume’, ‘Sapporo’ and ‘Shishito’. Error bars indicate the standard error.

Brix percentage

In 2018, ‘Takanotsume’ plants grown in soil treated with 100, 200 and 300 $g \cdot m^{-2}$ P fertilizer gave higher Brix percentages than plants grown in the 0 $g \cdot m^{-2}$ P fertilizer condition (Fig. 10). ‘Sapporo’ fruits also showed lower Brix percentages when grown in 0 P fertilizer than at other P fertilizer levels. The Brix percentage of ‘Habanero’ fruit showed a significant increase from 0 to 200 $g \cdot m^{-2}$ P but not between 200 and 300 $g \cdot m^{-2}$ P fertilizer treatment.

‘Takanotsume’, ‘Sapporo’ and ‘Shishito’ showed no significant differences in Brix, regardless of treatment in 2020 (Fig. 11).

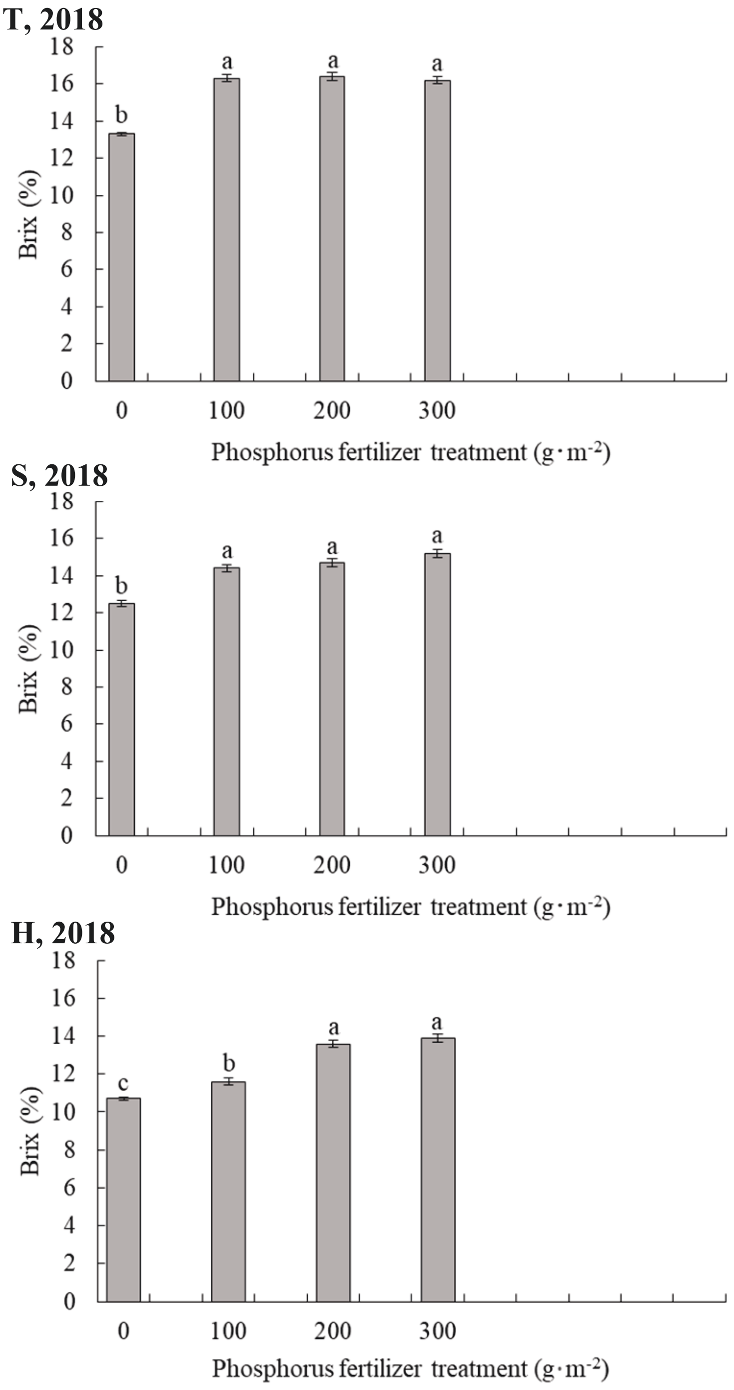


Fig. 10. Brix% of 'Takanotsume' (T) and 'Sapporo' (S) and 'Habanero' (H) chili pepper fruits in 2018. Different lower-case letters (a, b and c) indicate significant differences between treatments (Tukey's pairwise test, $p<0.05$) of 'Takanotsume', 'Sapporo', and 'Habanero'. Error bars indicate the standard error.

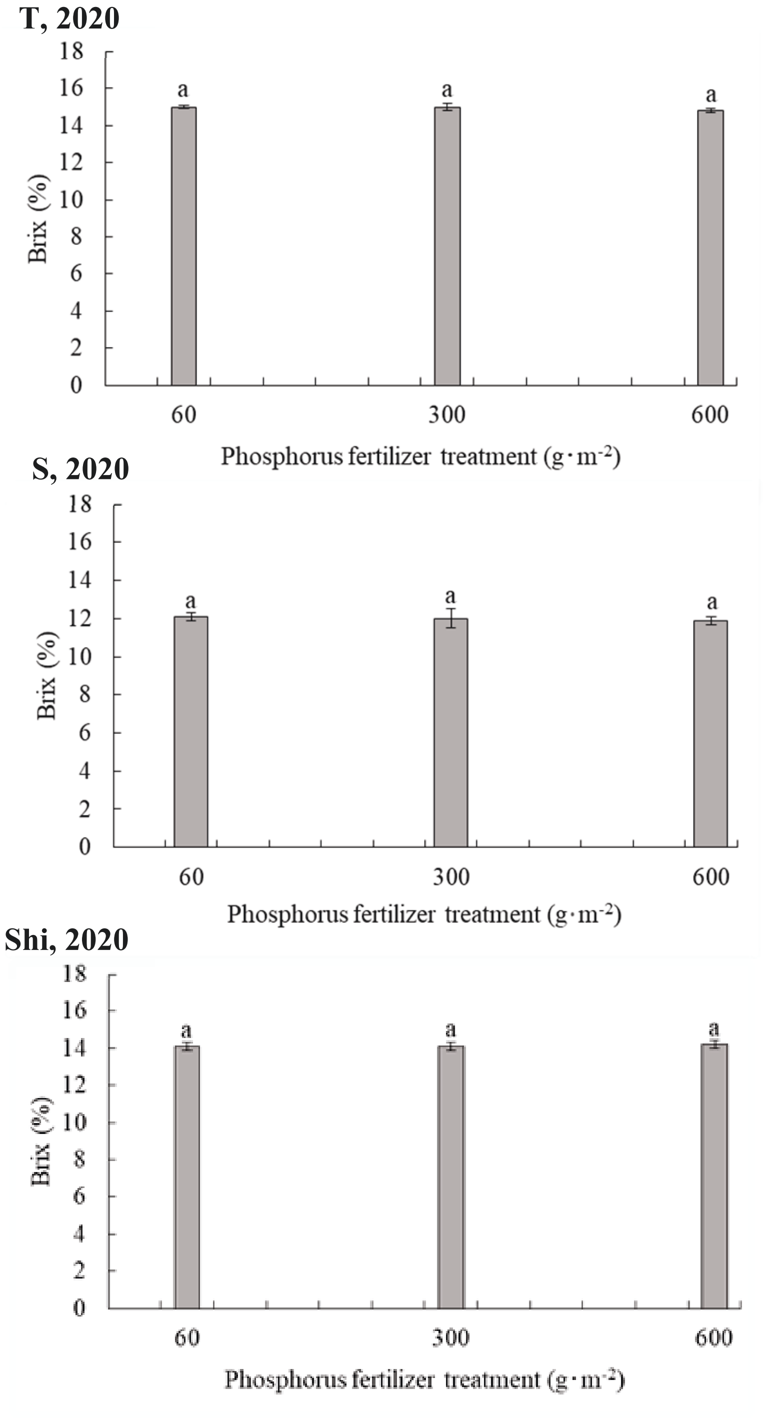


Fig. 11. Brix% for 'Takanotsume' (T) and 'Sapporo' (S) and 'Shishito' (S) chili pepper fruits in 2020. Different lower-case letters indicate significant differences between treatments (Tukey's pairwise test, $p<0.05$) of 'Takanotsume', 'Sapporo' and 'Shishito'. Error bars indicate the standard error.

Discussion

In the experiment of Kitamura et al. (2009) showed that excess N in the soil is unlikely to affect the capsaicinoid content during chili pepper cultivation because the application of excess N fertilizer has a negative effect on plant roots. On the other hand, P likely affects the capsaicinoid content of chili pepper because even an excess of P in the soil has no adverse effect on growth of plant, unlike N. (Foth, 1983)

Bajaj (1979) reported that capsaicinoid content increased with increasing amount of P applied, but the increase was not significant beyond a certain amount of applied P. On the other hand, Sega (1972) reported that the capsaicinoid content decreased with increasing application of P although no test of statistical significance was shown. Murugan (2001) found no significant difference in capsaicinoid content with P application probably because the number of test plots was small.

Then, Kitamura et al. (2009) showed a negative correlation between residual P in the soil and capsaicinoid content in the fruit, which indicates that excess P fertilizer levels had a negative effect on fruit pungency. There was no significant difference in capsaicinoid content in the 2008 results with various levels of P application (15-75g·m^{-2}) (Kitamura et al., 2009). The results of Kwawaguchi, personal contacts from 2010 and 2011, when the fertilizer application rate was subdivided and expanded up to 240 g·m^{-2} in 2010, showed that capsaicinoid content increased with increasing P application. Subsequently, when more than 120 g·m^{-2} was applied, the capsaicinoid content showed a decreasing trend, but no significant difference was observed. A similar trend was observed in 2011, although it was not statistically significant (Kwawaguchi, personal contacts). Even though, there was a significant difference in capsaicinoid content in 2010, no significant difference in capsaicinoid content in 2011 according to the results. When excess P application was tested in 2018 Experiment 1, the capsaicinoid content was significantly lower in the test plot containing the highest amount of P applied (300g·m^{-2}).

Taken collectively, the results suggest that the capsaicinoid content increases with an increasing amount of P application and decreases when P is in excess, which would be also in agreement with all the previous reports (Sega, 1972; Bajaj, 1979; Murugan, 2001).

In Experiment 1, capsaicinoid content of ‘Takanotsume’ is higher in fruit harvested in autumn than summer, but that of ‘Habanero’ is higher in fruit harvested in summer than autumn. Different plant species have different coping mechanisms against different stress conditions. (Chen and Murata, 2002). During the present experiment, plants have to face different temperatures in the summer and the autumn which is also a stress towards the chili plants. The susceptibility of plants to high temperature differs according to genotypes and also the developmental stages (Wahid et al., 2007). This might be the reason for observing different levels of capsaicinoid in the present experiment.

Lycopene is the most prominent carotenoid in tomatoes (Beecher, 1998). Experiments on tomatoes by Liu et al. (2011) showed that P affects the nutritional value of tomatoes by affecting lycopene content, and excessive P application degrades fruit quality by lowering the lycopene content. This situation is similar to that for capsaicin in chili peppers.

Excess P indirectly affects plant growth by reducing Fe, Mn and Zn uptake (McCauley et al., 2011). If these micronutrients also affect the capsaicinoid synthesis pathways, the capsaicinoid content may be reduced when P is added in excess. In the future, the effects of these micronutrients on capsaicinoid synthesis should be investigated.

Mycorrhiza is symbiotic fungi associated with plant roots that enhance the uptake of water and mineral nutrients, especially phosphate (Hogg, 2013). In pepper production, this mycorrhizal association greatly increases pepper yield and disease tolerance, and accelerates maturity (Yilma, 2019). Higher available soil P levels and fertilizer application may reduce mycorrhizal association (Grant et al., 2005). Kithamura et al. (2009) reported low capsaicinoid levels when a high amount of available P fertilizer was present in the soil samples. Thus, excess P fertilizer in the soil could reduce chili pepper plant P uptake, negatively affecting capsaicinoid biosynthesis and reducing the capsaicinoid content. It remains unknown how P affects capsaicinoid synthesis, but P is an essential element in compounds such as ATP, NADPH, nucleic acids, sugar phosphates, and phospholipids, all of which play important roles in photosynthesis (Hammond and White, 2008). Further studies should examine in more detail whether P application affects the capsaicinoid synthesis pathways.

It has been demonstrated that the ready availability of nutrients has a significant positive effect on tomato quality, color, and acceptability (Kimball and Mitchell, 1981). Furthermore, Lacatus et al. (1994) reported that K and P availability have a positive effect on fruit sugar and acid content in tomatoes. In our experiments conducted in 2018 and 2020, high P fertilizer treatment was also shown to have a positive effect on fruit glucose and total sugar content in chili pepper.

P deficiency in the leaf leads to leaf senescence (Usuda and Mathis, 1995) and reduces photosynthetic activity, and the resulting decrease in leaf photosynthetic activity reduces sugar transport to the fruit (Sanches et al., 1990). P is a major contributor to sugar transport from the leaf to the fruit (Marschner, 1995), possibly increasing glucose and total sugar content when the P fertilizer level is increased.

A hydroponic melon experiment conducted by Ben-Oliel and Kafkafi (2002) showed that an increase in P in the nutrient solution significantly increased fruit total soluble solids in melons. Previous experiments with tomatoes also showed that an increase in P and K significantly increased total soluble solids and acidity (Bagal et al., 1989).

Previous reports showed an inverse tendency between the glucose and total sugar content in chili pepper under drought (Rathnayaka et al., 2021b), salinity (Rathnayaka et al., 2021a) and temperature stress conditions (Chapter IV). Low glucose content and high total sugar content were reported during the stress conditions. However, high glucose content and low total sugar content were reported in control conditions of the same experiment. In contrast, the present experiment indicated that both glucose and total sugar content increased when the P fertilizer level increased.

Our experiments in 2018 with 'Takanotsume' and 'Sapporo' showed that Brix increased when the P fertilizer level increased from 0 to 100 $g \cdot m^{-2}$ and the Brix for 'Habanero' increased significantly when the soil was treated from 0 to 200 $g \cdot m^{-2}$ P fertilizer. In other words, as to all chili varieties were grown in 2018, and 0 $g \cdot m^{-2}$ P fertilizer treatment showed reduced Brix values compared with plants grown in the presence of P fertilizer.

Another tomato experiment showed that water management, K rate, and the interaction between water and K had significant effects on soluble solid content (Brix%), whereas P application with combinations of water and K had no significant effect on Brix % (Liu et al., 2011). In our 2020 experiment, the Brix parentage showed no increasing or decreasing pattern for fruit grown in soil treated with 60, 300 or 600 $g \cdot m^{-2}$ P, showing that the lack of P fertilizer negatively affects the Brix value and adequate P gives rise to high-quality chili pepper fruits.

Finally, pungency was enhanced when P fertilizer is applied appropriately but weakened when over-applied. Larger amounts of P fertilizer may increase sweetness but not when over-applied. Therefore, before planting chili pepper, it is important to measure the soil-available P level and avoid adding excess P fertilizer to obtain good quality chili fruits.

Chapter III

Excess Phosphorus Reduces the Pungency and Expression of Capsaicinoid Biosynthesis Genes in Chili Pepper (*Capsicum annuum* L.)

Abstract

The present study was conducted to determine the effects of excess phosphorus fertilizer application on a number of seeds, placental septum weight, and capsaicinoid content in ‘Takanotsume’, ‘Sapporo Oonaga Namban’ and ‘Shishito’ (only used in 2020) chili pepper varieties under different phosphorus fertilizer treatments in 2020 (60, 300 and 600 $g{\cdot}m^{-2}$) and in 2021 (100 and 600 $g{\cdot}m^{-2}$). Furthermore, this research is also to determine the relative expression levels of capsaicinoid biosynthesis genes in 2021. With the increasing amount of P fertilizer added, the number of seeds and placenta dry weights did not change, but the capsaicinoid content was significantly lower in all varieties except for ‘Shishito’. Relative expression levels of 18 capsaicinoid biosynthesis genes were tested using a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in 2021. The genes were divided into four groups based on their expression patterns. Group 1 showed a higher gene expression of *Pun1*, *pAMT*, *ACL* and *CaKR1* genes in plants grown in 100 $g{\cdot}m^{-2}$ P-treated soil for both cultivars at 20 days after flowering (DAF). Group 2 genes, *WRKY9, BCKDH, KAS I, CaMYB31, HTC, KAS III and BCAT* showed a higher expression in ‘Takanotsume’ under the 100 $g{\cdot}m^{-2}$ P fertilizer treatment at 20 DAF. Group 3 comprised *ACS*, *FAT* and *COMT*, showed higher expression in at least one variety grown in 100 $g{\cdot}m^{-2}$ P fertilizer at 30 DAF. The increased expression of groups 1, 2 and 3 genes induced increases in the pungency of chili peppers grown in 100 $g{\cdot}m^{-2}$ P fertilizer.

Introduction

Capsicum annuum L. is the most widely grown spice globally (Bosland, 1994), from the tropics to temperate zones, and is one of five domesticated chili pepper species (Bosland and Votava, 1999). Capsaicinoids are responsible for the pungent taste of chili and accumulate in the vesicles and vacuoles of epidermal cells of the placenta (Bosland et al., 2015) mostly between 20 and 30 days after flowering (DAF) (Iwai et al., 1979). They are mainly used in the food, pharmaceutical and medical industries (Naves et al., 2019). The most prominent capsaicinoids are capsaicin and dihydrocapsaicin, which together represent 90% of the total capsaicinoids in chilies (Kozukue et al., 2005). They are biosynthesized through a combination of the phenylpropanoid and branched-chain fatty acid pathways (Aza-González et al., 2011). The former pathway produces vanillylamine while the latter one produces 8-methyl-6-nonenoyl-CoA, and are condensation-coupled and biosynthesized into capsaicinoids by the *Pun1* expression (Ogawa et al., 2015).

Plant genotypes greatly affect the capsaicin and dihydrocapsaicin content (Tripodi et al., 2018). Pungency is also affected by environmental factors such as drought (Rathnayaka et al., 2021c), salinity stress (Rathnayaka et al., 2021b), parthenocarpy (Kondo et al., 2020; Kondo et al., 2021), temperature (Chapter IV), light (Gangadhar et al., 2012) and fertilizer condition (Buczkowska et al., 2013).

Nitrogen (N) fertilizer significantly increases plant growth and capsaicinoid content, whereas potassium (K) fertilizer has no positive effect on growth or productivity in chili cultivars (Medina-Lara et al., 2008). The phosphorus (P) application significantly decreases the dry matter content of chili peppers and greatly increases growth and yield

(Emongor and Mabe, 2012). Murugan (2001) discovered that the N application increases the capsaicin content in green, ripe and dry chilies but no significant differences with the P application (30 and 60 $Kg \cdot ha^{-1}$ P_2O_5) or with different sources of P. Bajaj (1979) reported that capsaicinoid content increases with increasing amounts of P at different levels of N, but the increase was not significant and the capsaicinoid content of the chilies under different P levels, remained unclear.

Japanese agricultural soil has been enriched by long-term excess P fertilizer application (Altansuvd et al., 2014). The authors revealed that the lack of (0 $g \cdot m^{-2}$ P) or excess (300 $g \cdot m^{-2}$ P) P in the soil reduced the capsaicinoid content in 'Takanotsume', 'Sapporo Oonaga Namban' (hereinafter referred to as 'Sapporo') and 'Habanero' pepper fruits, with 100 $g \cdot m^{-2}$ P fertilizer added providing the highest values in 'Sapporo' and 100 $g \cdot m^{-2}$ and 200 $g \cdot m^{-2}$ P levels yielding the highest values in 'Takanotsume' and 'Habanero' (Chapter I). The objective of the present study was to determine the relationship between the excess P on capsaicinoid content, number of seeds, placental septum weight, and on the expression level of capsaicinoid biosynthesis genes.

Material and Method

The field experiment was conducted at the Alpine Field Research and Education Center, Faculty of Agriculture, Shinshu University, Minamiminowa, Nagano, Japan (a.s.l. 733 m) in 2020 and 2021. Three *C. annuum* local varieties ('Takanotsume', 'Sapporo' and 'Shishito') were grown, using three different levels of P fertilizer application (60, 300 and 600 $g \cdot m^{-2}$) in 2020 (Experiment 1), and 'Takanotsume' and 'Sapporo' were grown, using two different levels of P fertilizer application (100 and 600 $g \cdot m^{-2}$) in 2021(Experiment 2). 'Takanotsume', 'Sapporo' and 'Shishito' seeds were purchased from Takii (Kyoto, Japan), Tsurushin (Matsumoto, Japan) and Sakata Seeds (Kanagawa, Japan), respectively.

Seedlings, approximately 100-150 mm in height were transplanted into clay pots (18 cm in diameter, 3.5 L), filled with commercial potting medium (Nae-Ichiban; Sumitomo Forestry Landscaping Co., Ltd., Tokyo, Japan) and placed in a greenhouse with 50% shading. The first set of flower buds was removed after applying the treatments. Commercial potting medium usually contains 60 $g \cdot m^{-2}$ equivalent of P fertilizer. Therefore, the 60 $g \cdot m^{-2}$ treatment did not require the addition of any P fertilizer and was used as the standard fertilizer treatment in 2020. The other P fertilizer treatments in 2020 and 2021 (100, 300 and 600 $g \cdot m^{-2}$) were prepared by adding 'Multi Phosphate' (available P: 35%) to the fertilizer. We previously determined that the peak capsaicinoid content was obtained using 100 $g \cdot m^{-2}$ P and 200 $g \cdot m^{-2}$ P fertilizer (In Chapter II). Therefore, 100 $g \cdot m^{-2}$ P fertilizer application was used as a control (C) and 600 $g \cdot m^{-2}$ P as excess (E) P fertilization in 2021.

Water was applied as required depending on the daily temperature and weather conditions. If the temperature was above 30 °C, 130 mL of water was added to the soil 3 times a day. On normal sunny days, if the day temperature was below 30 °C, the same amount of water was applied twice a day. Water was applied ones a day on rainy days. Other crop management practices followed standard recommendations for growing peppers in Japan. During the fruiting stage, the plants were supported with by plastic poles.

Experiment 1 (conducted in 2020)

Experiment 1 was conducted from the 15th of May to the 30th of September, 2020. The maximum average temperature was 35.0 °C, with the minimum at 20.5 °C. Fruits were harvested on a pre-decided day (25th of September) and divided into two categories, depending on the appearance of the pericarp. Those that are fully red are considered late mature fruits and those beginning to turn dark green are regarded as early mature ones. Each P fertilizer treatment was carried out for five plants. One fruit per plant was harvested at the early mature and late mature stages, and the number of seeds were counted and dry weights of the placenta septum were measured. Each dried placenta septum was used for the analysis of the capsaicinoid content.

Experiment 2 (conducted in 2021)

Experiment 2 was conducted from the 1st of June to the 17th of September, 2021. The maximum average temperature was 33.5 °C, with the minimum average at 19.0 °C. Each P fertilizer treatment was carried out for five plants. Selected flowers were tagged when just opening, and one fruit per plant was harvested at 20 and 30 DAF. The number of seeds of each fruit were counted. The placenta septum was separated from each fruit and divided into two equal parts. One half of each placenta was used for measuring the dry weight with capsaicinoid analysis afterwards while the other half was kept at −80 °C for RNA extraction.

Capsaicinoids extraction and analysis

Placenta septum samples, used for capsaicinoid analysis, were freeze-dried using a freeze-dryer (Islay FDU – 2000, Rikakikai Co., Ltd., Tokyo, Japan) for 24 hours, placed in collection tubes with a stainless-steel ball, and pulverized, using a Micro Smash TM MS-100 (TOMY SEIKO Co., Ltd., Tokyo, Japan). For capsaicinoid extraction, 5 mL of acetone was added to each sample and then left for 5 min. The top layer was transferred to a round bottom flask. 2 mL ethyl acetic acid was added to the remaining sedimented powder and transferred to the same flask. The extract was evaporated to dryness at 40 °C using a rotary evaporator (N-1100; Tokyo Rikakikai). The extracted capsaicinoid was dissolved in 5 mL methanol, then 10 µL of the extract was used to analyze the capsaicinoid content in each sample, using high-performance liquid chromatography (HPLC) (YMC-Pack ODS-A, 50 × 3.0 mm, Shimadzu Corporation, Kyoto, Japan) with a column temperature of 40 °C, a mobile phase of 65% methanol, a flow rate of 1 $mL \cdot min^{-1}$ and monitoring at 280 nm. Capsaicinoid concentration was calculated based on the peak area of capsaicin and dihydrocapsaicin.

RNA isolation and qRT-PCR of capsaicinoid biosynthesis genes

Two P fertilizer treatments (100 $g \cdot m^{-2}$ and 600 $g \cdot m^{-2}$) were used to grow 'Takanotsume' and 'Sapporo' fruits, after which total RNA was extracted from the frozen placenta septum (-80 °C) using a RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Samples were treated with DNase to remove genomic

DNA using a DNase Max® Kit (Qiagen). The concentration of RNA was measured using a BioSpec-nano (Shimadzu Corporation). RNA was converted to cDNA by reverse transcription PCR (Applied Biosystems ProFlexTM Base) using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Massachusetts, USA).

Eighteen capsaicinoid biosynthesis genes (Fig. 1) were analyzed as target genes by qRT-PCR using a Step One Real-Time PCR System (Applied Biosystems). Actin was used as the reference gene according to Tanaka et al. (2017), Koeda et al. (2019), Han et al. (2018) and Kondo et al. (2021). The primers used for gene amplification are shown in Table 1 and a PowerUpTM SYBR Green Master Mix (Applied Biosystems) was used as the PCR mixture. A thermal cycle according to Tanaka et al. (2017) was used (98 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 68 °C for 60 s). The relative expression level was calculated based on the comparative threshold cycle (Ct) method.

Table 1. Primers used for qRT-PCR.

Gene name		Primer sequence (5'-3')	Reference
PAL	F:	CAACAGCAACATCACCCCATGTTTGC	Tanaka et al., (2017)
	R:	GCTGCAACTCGAAAAATCCACCAC	
C4H	F:	CTTGGTTAACGCTTGGTGGT	Tanaka et al., (2017)
	R:	CCGAATGGAAGGAATCTGAA	
4CL	F:	GGACCGATTGAAGGAATTGA	Tanaka et al., (2017)
	R:	GGACAACAGCAGCATCAGAA	
C3H	F:	GCCATCTTCTGCACCATTTT	Tanaka et al., (2017)
	R:	GGCCTGTAATGGAGTCCTCA	
HCT	F:	ATGCAGGGATGAAGATGGAC	Tanaka et al., (2017)
	R:	TAATCAACGGCCGGAATAAG	
COMT	F:	CCTGCGAATGGAAAAGTGAT	Tanaka et al., (2017)
	R:	TCTTTGCCTCCTGGGTTATG	
pAMT	F:	ATACTCAAAGAGGGGCCTGAAACAG	Tanaka et al., (2017)
	R:	TTCCAAATCCACATACCACCTCATC	
BCAT	F:	CAAGGAAGGAACAGCACCAT	Tanaka et al., (2017)
	R:	TCGCCTTTGCTTTCTTCATC	
BCKDH	F:	CGGATGGCTGTTGAAGAAGT	Tanaka et al., (2017)
	R:	CTCCTTTGCAGCTTCTACGC	
KAS I	F:	GTGTACAAATGCCAGCAAGCTCTG	Tanaka et al., (2017)
	R:	GATTCCACTTTGTCCCTCGAGAAG	
KAS III	F:	ATGTTGACTGGACAGATAGAGGGA	Zhu et al., (2019)
	R:	AGAGTAAGAGGAAGTTTTTGGTGG	
CaKR1	F:	GCTTGGGATGTGTTTGGTAGG	Koeda et al., (2019)
	R:	TGCTCCTCTTAGGTTCGTTTTG	
ACL	F:	GCCACTCGTCGCCTCAGTAT	Tanaka et al., (2017)
	R:	GCGCAGCAAACTTGGACTCT	
FAT	F:	CAATGTTGTCTCGGGGGAGTTTTC	Tanaka et al., (2017)
	R:	CTCTCTCTCTCATTAGTAGCTACAGC	
ACS	F:	TGGCTCAGCTGAATTTGTTG	Tanaka et al., (2017)
	R:	TAACCCGTGAACGTGAAACA	
Pun1	F:	ATCTCAACGAGTGCGTACAGAAAAGACT	Tanaka et al., (2017)
	R:	GTGAACCAACTTTGATGGTAGCATTGAT	
WRKY9	F:	ATGTGAATCGGCTACAATGAA	Kondo et al., (2021b)
	R:	GTGCAACGGTAGTAAGCTC	
CaMYB31	F:	GACGAAAATGGAATGAAGAAGG	Han et al., (2018)
	R:	AGCATGGAGGTTCAAGATGATT	
Actin	F:	AGCAACTGGGACGATATGGAGAAG	Tanaka et al., (2017)
	R:	AAGAGACAACACCGCCTGAATAGC	

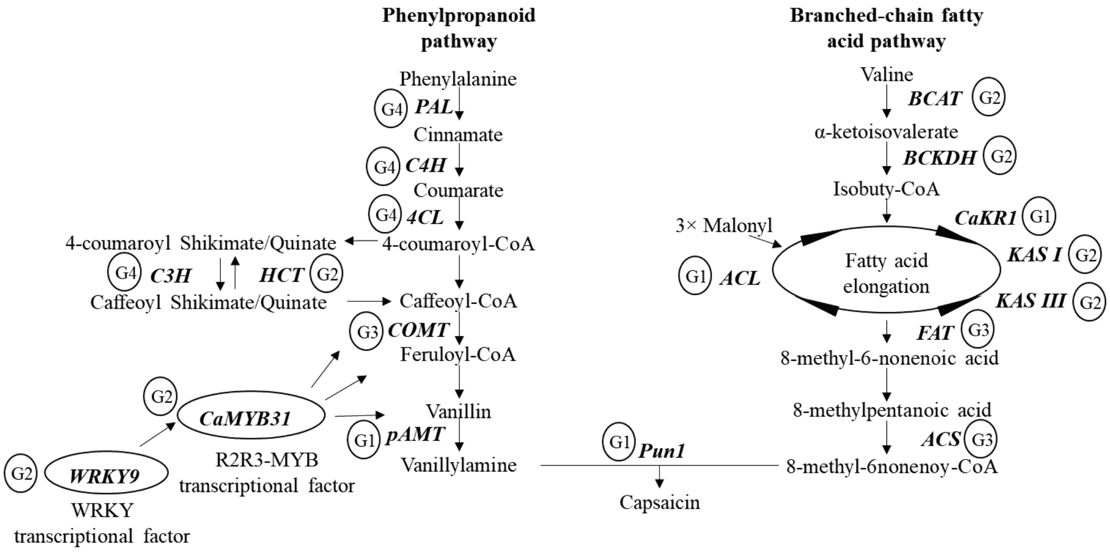


Fig. 1. The capsaicinoid biosynthesis pathway and the locations where the capsaicinoid biosynthesis genes analyzed in the present study are involved, except for *CaMYB31* and *WRKY9* (Modified from Arce-Rodríguez and Ochoa-Alejo, 2019; Koeda et al., 2019; Kondo et al., 2021). *PAL*: *phenylalanine ammonia lyase*, *C4H*: *cinnamate 4-hydroxylase*, *4CL*: *4-coumaroyl-CoA ligase*, *HCT*: *hydroxycinnamoyl transferase*, *C3H*: *coumaroyl shikimate/quinate 3-hydroxylase*, *COMT*: *caffeic acid O-methyltransferase*, *pAMT*; *putative aminotransferase*, *BCAT*: *branched-chain amino acid transferase*, *BCKDH*: *branched-chain α-ketoacid dehydrogenase*, *ACL*: *acyl carrier protein*, *KAS*: *ketoacyl-ACP synthase*, *CaKR1*: *ketoacyl-ACP reductase*, *FAT*: *acyl-ACP thioesterase*, *ACS*: *acyl-CoA synthetase*, and *Pun1*: *acyltransferase*. G1, G2, G3 and G4 respectively represent expression pattern groups 1, 2, 3 and 4 in Fig. 2.

Results

Number of seeds and placental septum weight

The 2020 experiment indicated that the average number of seeds and dry placenta weights (Table 2) did not show any significant difference in ‘Takanotsume’, ‘Sapporo’ and ‘Shishito’ plants grown in soil treated with 60, 300 and 600 $g{\cdot}m^{-2}$ P, whether they were harvested at an early mature or late mature stage.

The 2021 experiments involved 100 and 600 $g{\cdot}m^{-2}$ P fertilizer treatments, also with no significant difference in the average number of seeds and dry placenta weights (Table 3) in ‘Takanotsume’ and ‘Sapporo’ fruits at 20 and 30 DAF.

Capsaicinoid content

The 2020 results showed that the capsaicinoid concentration in ‘Takanotsume’ and ‘Sapporo’ plants, grown using 60 $g{\cdot}m^{-2}$ P fertilizer was significantly higher than that of those grown using 600 $g{\cdot}m^{-2}$ P fertilizer at either the early or late mature harvesting stages. Whereas ‘Shishito’ showed no significant difference regardless of the P fertilizer treatment at any harvesting stage (Table 2).

The 2021 experiment indicated that the capsaicinoid concentration was significantly higher in ‘Takanotsume’ and ‘Sapporo’ cultivars, subjected to the 100 $g{\cdot}m^{-2}$ P fertilizer treatment compared to the 600 $g{\cdot}m^{-2}$ (excess) P fertilizer treatment at both 20 and 30 DAF (Table 3).

Table 2. Average number of seeds, placental septum dry weight and capsaicinoid content of placenta septum in ‘Takanotsume’, ‘Sapporo’ and ‘Shishito’ chili peppers grown under different phosphorus fertilizer treatments in 2020.

Varieties	P treatment ($g \cdot m^{-2}$)	Average number of seeds		Placental septum dry weight (g)		Capsaicinoid content of placenta septum (µg·g-1 DW)	
		Mature stage		Mature stage		Mature stage	
		Early	Late	Early	Late	Early	Late
Takanotsume	60	13.4 ± 8.5 a	28.2 ± 10.7 a	0.0121 ± 0.0012 a	0.0134 ± 0.0041 a	64839 ± 24962 a	65172 ± 16491 a
	300	18.0 ± 6.9 a	26.8 ± 5.2 a	0.0152 ± 0.0019 a	0.0124 ± 0.0037 a	37629 ± 17959 ab	54359 ± 9081 ab
	600	19.8 ± 3.2 a	21.0 ± 8.3 a	0.0158 ± 0.0039 a	0.0151 ± 0.0021 a	31966 ± 18066 b	27148 ± 27315 b
Sapporo	60	28.6 ± 9.5 a	32.0 ± 18.1 a	0.0285 ± 0.0096 a	0.0306 ± 0.0082 a	27718 ± 14519 a	30246 ± 13016 a
	300	34.0 ± 3.7 a	44.2 ± 18.8 a	0.0315 ± 0.0107 a	0.0316 ± 0.0085 a	23530 ± 6273 ab	24078 ± 13968 ab
	600	23.8 ± 11.3 a	40.0 ± 11.6 a	0.0246 ± 0.0064 a	0.0293 ± 0.0083 a	6741 ± 5708 b	9877 ± 3054 b
Shishito	60	61.6 ± 16.3 a	40.0 ± 10.1 a	0.0681 ± 0.0242 a	0.0410 ± 0.0109 a	150 ± 45 a	444 ± 450 a
	300	52.2 ± 16.8 a	43.0 ± 9.5 a	0.0588 ± 0.0255 a	0.0365 ± 0.0112 a	124 ± 75 a	204 ± 226 a
	600	34.8 ± 22.0 a	34.0 ± 16.3 a	0.0408 ± 0.0094 a	0.0434 ± 0.0159 a	123 ± 93 a	72 ± 43 a

Values (means ± standard deviation, n=5) followed by the same letter in a column of each chili varieties are not significantly different at 5 % level by Tukey's pairwise test.

Table 3. Average number of seeds, placental septum dry weight and capsaicinoid content of placenta septum in ‘Takanotsume’ and ‘Sapporo’ chili peppers grown under different phosphorus fertilizer treatments in 2021.

Varieties	P treatment (g·m^{-2})	Average number of seeds		Placental septum dry weight (g)		Capsaicinoid content of placenta septum (μg·g^{-1} DW)	
		Harvesting date		Harvesting date		Harvesting date	
		20 DAF	30 DAF	20 DAF	30 DAF	20 DAF	30 DAF
Takanotsume	100	25.6 ± 7.3 ns	29.0 ± 6.0 ns	0.0061 ± 0.0026 ns	0.0111 ± 0.0063 ns	43256 ± 8247 **	48071 ± 11151 **
	600	25.8 ± 5.0	30.0 ± 6.2	0.0118 ± 0.0049	0.0145 ± 0.0033	20666 ± 9088	25845 ± 6098
Sapporo	100	32.8 ± 11.1 ns	33.0 ± 6.7 ns	0.0169 ± 0.0049 ns	0.0204 ± 0.0046 ns	6245 ± 1867 *	17720 ± 2690 ***
	600	36.0 ± 8.9	34.6 ± 9.5	0.0195 ± 0.0051	0.0224 ± 0.0030	2907 ± 1857	9363 ± 2170

Significant differences were analyzed using Student’s *t*-test. Values (means ± standard deviation, n=5) followed by ‘ns’ in a column of each chili variety are not significantly different, * represents the significance level at 5%, ** represents the significance level at 1% and *** represents the significance level at 0.1% for the same DAF and variety.

Expression analysis of capsaicinoid biosynthesis pathway genes

qRT-PCR analysis was conducted for 18 capsaicinoid biosynthesis target genes using placental septum from ‘Takanotsume’ and ‘Sapporo’ grown in 100 $g{\cdot}m^{-2}$ and 600 $g{\cdot}m^{-2}$ P fertilizer. Based on the expression patterns, the expression results were divided into four groups. *Pun1* (acyltransferase), *pAMT* (putative aminotransferase), *ACL* (acyl carrier protein) and *CaKR1* (ketoacyl-ACP reductase) genes comprised group 1 (Fig. 2), and a higher gene expression level was observed in the fruits of ‘Takanotsume’ and ‘Sapporo’, grown using control P fertilizer (100 $g{\cdot}m^{-2}$ P) than in the fruits of those grown, using excess P fertilizer (600 $g{\cdot}m^{-2}$ P) harvested at 20 DAF. Only the *pAMT* gene showed a higher expression level in 30 DAF ‘Takanotsume’ fruit.

The genes in group 2 were *WRKY9*, *BCKDH* (branched-chain α-ketoacid dehydrogenase), *KAS I* (ketoacyl-ACP synthase I), *CaMYB31*, *HTC* (hydroxycinnamoyl transferase), *KAS III* (ketoacyl-ACP synthase III) and *BCAT* (branched-chain amino acid transferase) (Fig. 2). Group 2 genes showed higher expression levels in ‘Takanotsume’ at 20 DAF grown using 100 $g{\cdot}m^{-2}$ P fertilizer compared to plants grown using 600 $g{\cdot}m^{-2}$ P fertilizer, and *BCAT* showed a higher expression level only in ‘Takanotsume’ at both 20 and 30 DAF.

Group 3 included *ACS* (acyl-CoA synthetase), *FAT* (ketoacyl-ACP reductase) and *COMT* (caffeic acid O-methyltransferase), and showed higher expression levels at 30 DAF in ‘Takanotsume’, grown in 100 $g{\cdot}m^{-2}$ P soil than plants in 600 $g{\cdot}m^{-2}$ P soil (Fig. 2). The *ACS* gene showed higher expression levels in ‘Takanotsume’ at 30 DAF and ‘Sapporo’ at 20 DAF, grown in 100 $g{\cdot}m^{-2}$ P soil compared to plants in 600 $g{\cdot}m^{-2}$ P soil.

COMT showed a higher expression level in ‘Takanotsume’ and ‘Sapporo’ at 30 DAF grown in the control P fertilizer conditions than in excess P fertilizer conditions.

Group 4 comprised *PAL* (phenylalanine ammonia lyase), *4CL* (4-coumaroyl-CoA ligase), *C4H* (cinnamate 4-hydroxylase) and *C3H* (coumaroyl shikimate/quinate 3-hydroxylase) and did not show any significant differences in gene expression for any of the cultivars at all DAFs, grown in either 100 $g{\cdot}m^{-2}$ or 600 $g{\cdot}m^{-2}$ P fertilizer-treated soil (Fig. 2).

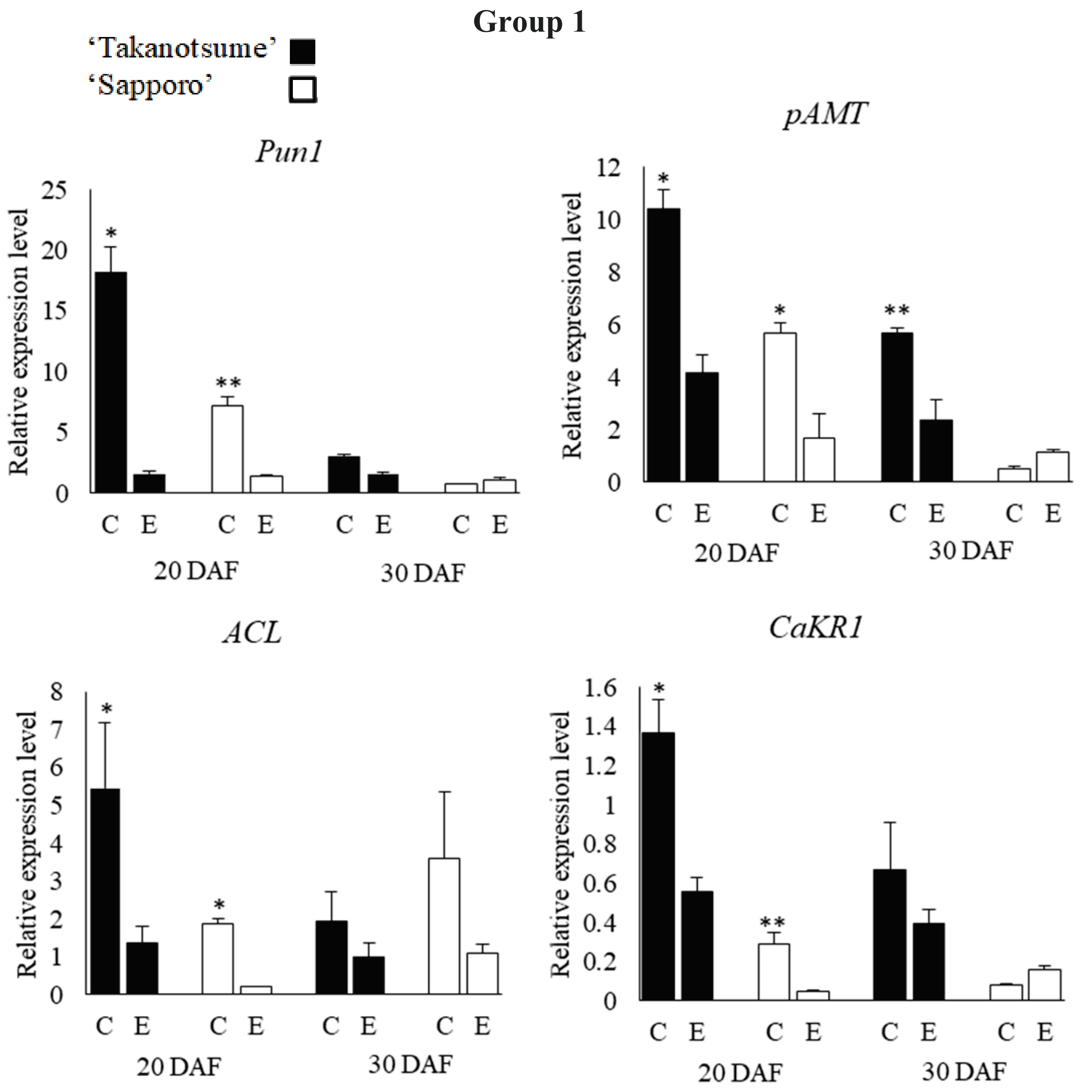


Fig. 2. Relative expression levels of 18 capsaicinoid biosynthesis genes in the placental septum of 'Takanotsume' and 'Sapporo' chili peppers cultivated under control [100 g·m^{-2} P fertilizer application, (C)] and excess [600 g·m^{-2} P fertilizer (E)] P conditions in 2021. Fruits were sampled at 20 and 30 days after flowering (DAF). Significant differences were analyzed using Student's *t*-test, * represents the significance level at 5%, ** represents the significance level at 1% for the same DAF and variety. Error bars indicate the standard error.

Group 2

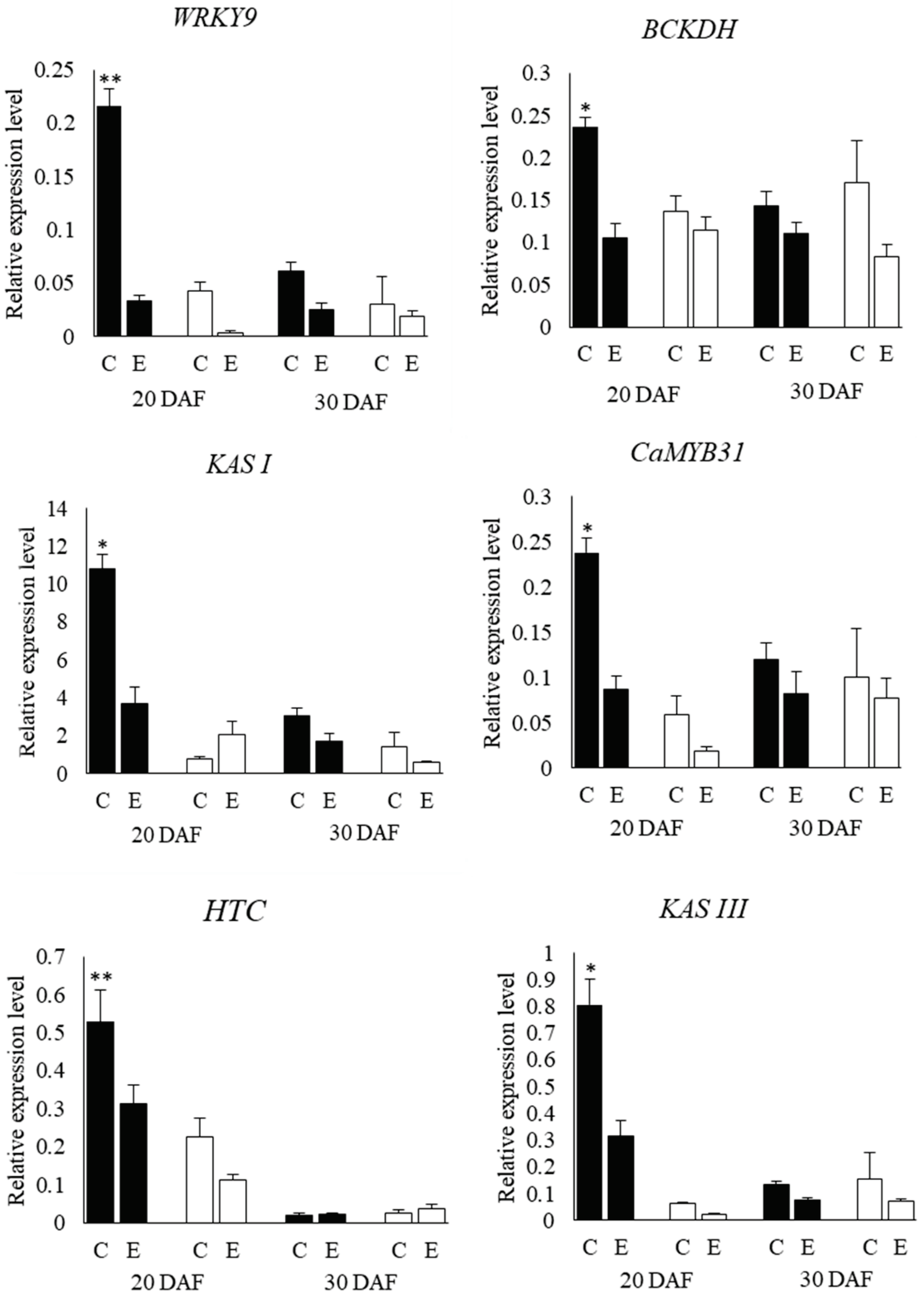


Fig. 2. Continued.

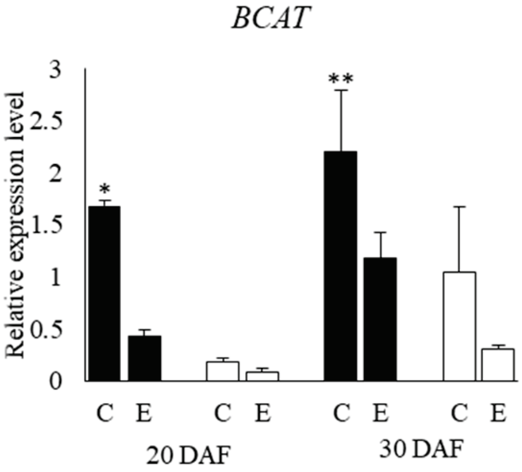


Fig. 2. Continued.

Group 3

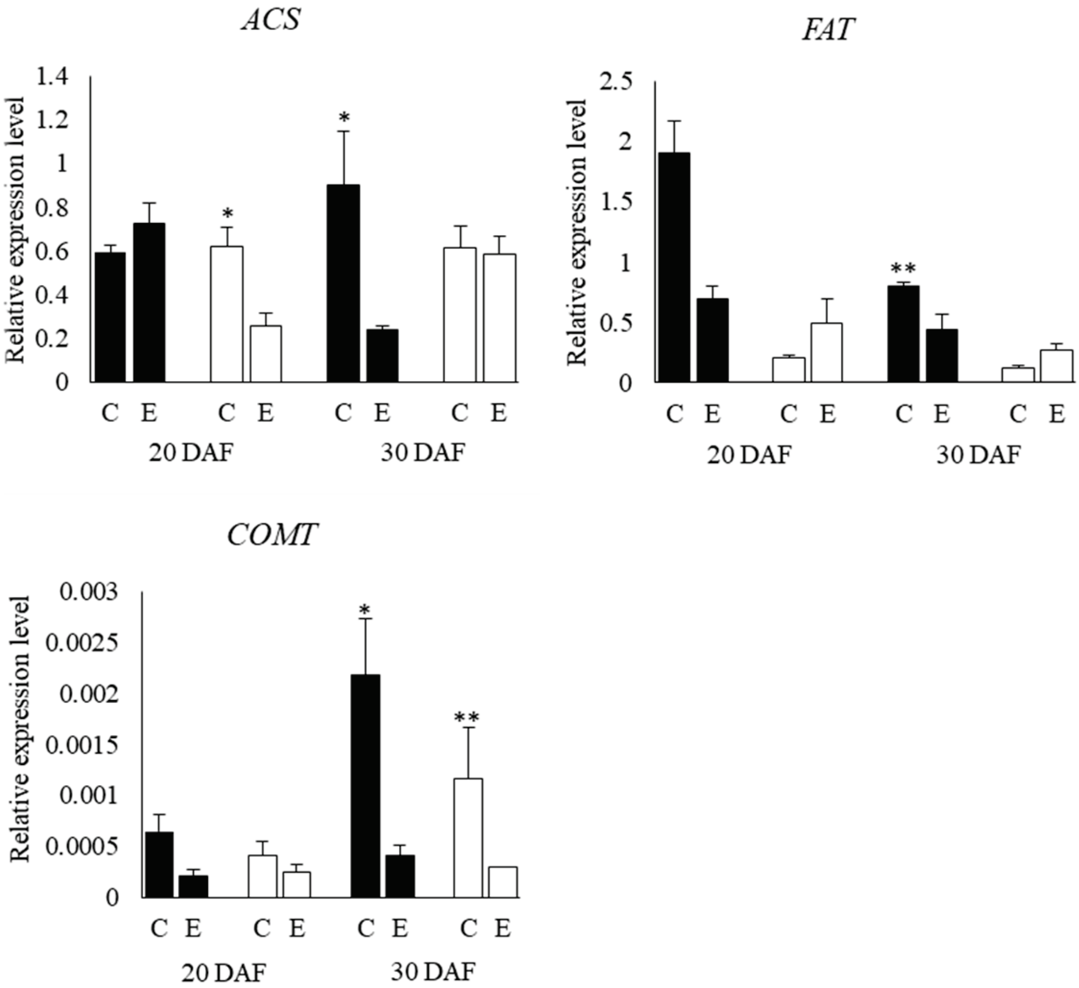


Fig. 2. Continued.

Group 4

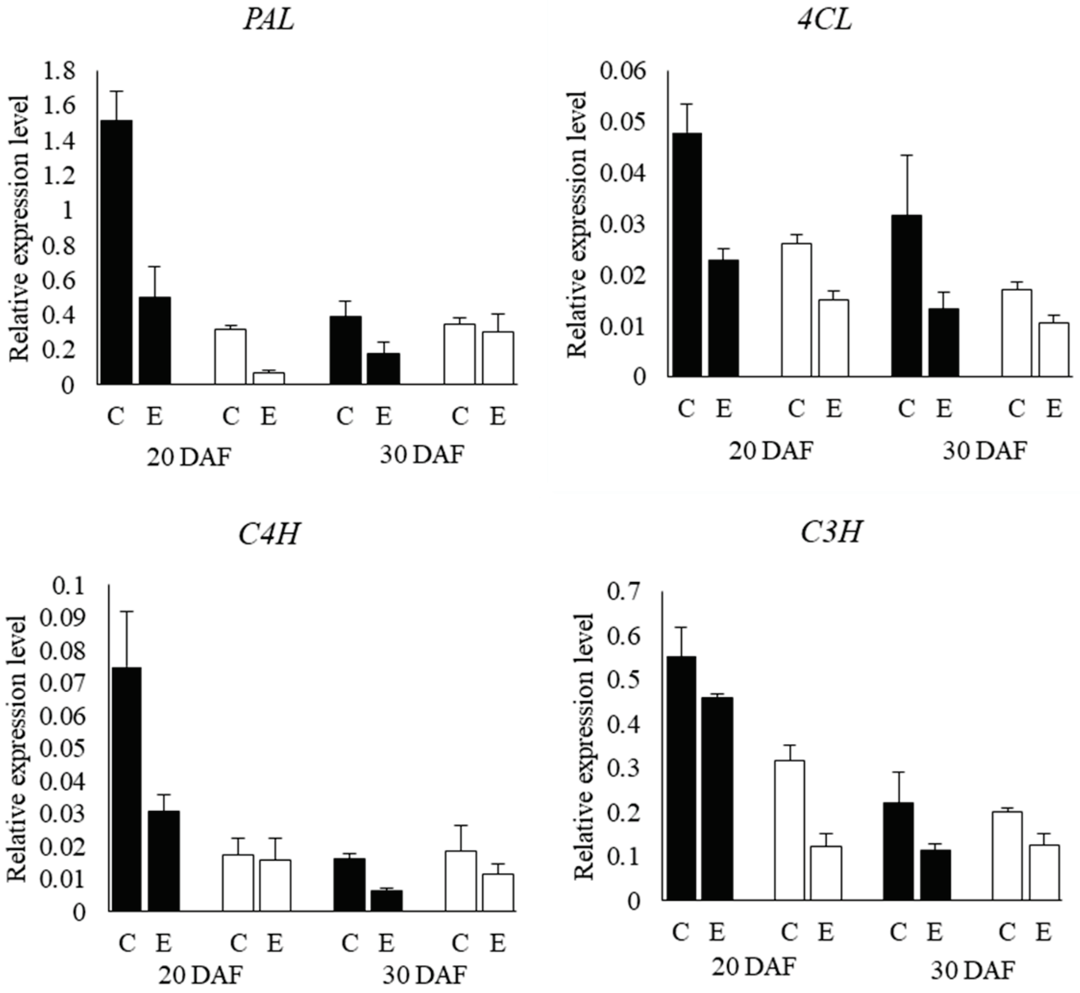


Fig. 2. Continued.

Discussion

The number of seeds and placenta dry weights of three chili varieties did not change with the amount of P fertilizer applied in 2020 and 2021 (Tables 2 and 3), but the capsaicinoid content of ‘Takanotsume’ and ‘Sapporo’ grown under excess (300 $g{\cdot}m^{-2}$) and extremely excess (600 $g{\cdot}m^{-2}$) P conditions were significantly lower than that grown under 60 $g{\cdot}m^{-2}$ and 100 $g{\cdot}m^{-2}$ P conditions, respectively at any harvesting stages. These results are compatible with the authors’ previous findings that the capsaicinoid content peaked in plants grown in soil treated with 100 $g{\cdot}m^{-2}$ and 200 $g{\cdot}m^{-2}$ P compared to those grown in the absence of added (0 $g{\cdot}m^{-2}$ P) and excess (300 $g{\cdot}m^{-2}$ P) fertilizer (Chapter II). However, the capsaicinoid content of ‘Shishito’ did not show any significant relationship with the different P fertilizer treatments at either early or late mature stages, which is partly because the level of content was comparatively very low in ‘Shishito’ than in ‘Takanotsume’ and ‘Sapporo’.

The physical defense of seeds is provided by the seed coat (lignin) and its chemical one is provided by capsaicin. Both lignin and capsaicin are phenols and are biosynthetically linked, resulting in a trade-off between the two (Tewksbury et al., 2008). According to Kondo et al. (2020), seedless parthenocarpy fruits generated by stigma excision and 2,4-D treatment increased pungency compared to naturally pollinated fruits that have a typical number of seeds. Drought stress increases the capsaicinoid content but gradually decreases the number of seeds and placenta septum weight (Rathnayaka et al., 2021c). Thus, the capsaicinoid content increases during parthenocarpy and drought stress because of the reduced number of seeds. The trade-off between the capsaicinoid and lignin biosynthesis resulted in increasing the capsaicinoid content, not because of the ability of synthesis of the capsaicinoid in the placental septum. In the present study, the seed number did not change in different P fertilizer treatments. However, the capsaicinoid content of plants grown in soil, treated with excess P (300 and 600 g·m^{-2} P) was significantly lower than those grown in soil treated with 60 and 100 g·m^{-2} P, respectively. This strongly suggest that the capsaicinoid synthesis ability of the placenta septum clearly decreases when excess P fertilizer is applied.

In the present study, qRT-PCR analysis was conducted for 18 capsaicinoid biosynthesis genes, and expression patterns were compared in 'Takanotsume' and 'Sapporo' grown in soil treated with 100 g·m^{-2} and 600 g·m^{-2} P fertilizer. The results were categorized into four groups based on expression patterns.

Group 1 comprised *Pun1*, *pAMT*, *ACL* and *CaKR1*, and these were significantly expressed at a higher level at 20 DAF in 'Takanotsume' and 'Sapporo' grown using 100 g·m^{-2} P fertilizer than in plants grown in 600 g·m^{-2} P fertilizer (Fig. 2). In the capsaicinoid synthesis pathway, *Pun1* connects both the phenylpropanoid pathway and the branched-chain fatty acid pathways and induces capsaicinoid accumulation (Stewart et al., 2005) (Fig. 1).

pAMT is involved in the final step of the phenylpropanoid pathway, which catalyzes the generation of vanillylamine from vanillin (Blum et al., 2003; Abraham-Jua´ rez et al., 2008). Vaninylamine and 8-methyl-6nonenoyl-CoA, a product of the fatty chain synthesis pathway, are condensation-coupled and biosynthesized into capsaicinoids by the *Pun1* expression. *Pun1* and *pAMT* are always highly expressed when there are high levels of capsaicinoid in various pepper cultivars (Ogawa et al., 2015). On the other hand, according to Koeda et al. (2015), the loss of functional *CaKR1* results in a single recessive non-pungency control gene when the plant lacks the loss-of-function recessive *Pun1* or *pAMT*. Koeda et al. (2019) successfully silenced *CaKR1* using a virus-induced gene silencing and observed a significant reduction of capsaicinoid accumulation in chili fruits. The inclusion of the *Pun1*, *pAMT*, and *CaKR1* genes in Group 1, which regulate pungency, indicates that these three also play an important role in changes in pungency in response to P fertilizer levels.

According to Rathnayaka et al. (2021a), ‘Sapporo’ and ‘Shishito’ cultivars exposed to drought stress conditions (resulting in plants with fruits with a high capsaicinoid content), express the *Pun1*, *pAMT*, *ACL*, *CaKR1*, *WRKY9*, *CaMYB31*, *FAT* and *KAS I* genes in chili fruits at 20 DAF. In the present study, plants grown in soil treated with 100 g·m^{-2} P (fruits with high capsaicinoid content) showed consistently high expression patterns, similar to those observed under drought stress conditions by Rathnayaka et al. (2021a), for the four genes *Pun1*, *pAMT*, *ACL* and *CaKR1* belonging to group 1.

The *Pun1*, *pAMT*, *KAS I* and *CaMY31* genes of ‘Shishito’ parthenocarpy fruits (seedless fruits with a high capsaicinoid content) showed higher expression levels than naturally pollinated fruits at 20 DAF (Kondo et al., 2020). In the present study, the fruits of plants grown using 100 g·m^{-2} P fertilizer showed the same high expression pattern for *Pun1* and *pAMT* as in the above experiment.

In group 2, the *WRKY9*, *BCKDH*, *KAS I*, *CaMYB31*, *HTC*, *KAS III* and *BCAT* genes were highly expressed only in the ‘Takanotsume’ variety at 20 DAF (Fig. 2). According to Rathnayaka et al. (2021a), under drought stress conditions *WRKY9*, *CaMYB31* and *KAS I* gene expression is significantly higher in both ‘Shishito’ and ‘Sapporo’, but *BCKDH* and *KAS III* are highly expressed only in ‘Shishito’ at 20 DAF. The *WRKY9*, *CaMYB31* and *KAS I* expressions are patterns of ‘Sapporo’ in the study by Rathnayaka et al. (2021a) differ from those in the present investigation because these three genes were not significantly expressed in ‘Sapporo’ at 20 DAF when grown in soil treated with 100 g·m^{-2} P fertilizer.

CaMYB31 and *KAS I* were previously shown to be highly expressed in parthenocarpy fruit at 20 DAF, as in research, but *BCKDH* and *HTC* did not show any specific expression in fruits harvested at 20, 35 and 50 DAF (Kondo et al., 2020), in contrast to the this study.

The *BCAT* gene is highly expressed in ‘Takanotsume’ at 20 and 30 DAF (Fig. 2). According to Tanaka et al. (2017), this gene showed a higher relative expression rate in a pungent variety than in a non-pungent one. ‘Takanotsume’ is more pungent than ‘Sapporo’, possibly explaining why the fruit from plants grown in 100 g·m^{-2} P-treated soil contained a higher expression level of *BCAT* in ‘Takanotsume’. *BCAT* in plants grown under drought stress conditions is highly expressed in ‘Shishito’ at 20 DAF and ‘Sapporo’ at 30 DAF (Rathnayaka et al., 2021a). *WRKY9* plays a crucial role in inducing the transcription factor *MYB31*, which is encoded by *CaMYB31* and expressed in *C. chinense*, therefore increasing the capsaicinoid content (Zhu et al., 2019). The present results suggest that *WRKY9* and *CaMYB31* induced increased capsaicinoid content not only in extremely spicy cultivars but also in the spicy cultivars of *C. annuum* when grown in soil treated with 100 g·m^{-2} P.

Group 3 genes *ACS*, *FAT* and *COMT* have higher expression levels at 30 DAF in ‘Takanotsume’ plants grown using 100 g·m^{-2} P fertilizer than those grown with 600 g·m^{-2} P fertilizer. The *ACS* gene was highly expressed in ‘Sapporo’ at 20 DAF and ‘Takanotsume’ at 30 DAF in plants grown using 100 g·m^{-2} P fertilizer. *ACS* in ‘Sapporo’ plants at 20 DAF grown in drought stress conditions showed a significantly high gene expression as seen in the present study and 'Shishito' at 30 DAF (Rathnayaka et al., 2021a). The expression level of *ACS* increased from 20 to 50 DAF but no specific gene expression was observed in parthenocarpy fruit (Kondo et al., 2020). *FAT* showed a significantly higher expression level in 'Takanotsume' at 30 DAF in plants grown under 100 $g \cdot m^{-2}$ P treatment conditions (Fig. 2). *FAT* is expressed at higher levels in parthenocarpy 'Shishito' plants at 20 DAF than in naturally pollinated 'Shishito' and in 2, 4-D treated 'Shishito' ones. It was expressed more in naturally pollinated 'Takanotsume', used as a control at 20 DAF (Kondo et al., 2021). Under drought stress, *FAT* is highly expressed in 'Shishito' and 'Sapporo' at 20 DAF (Rathnayaka et al., 2021a). According to Rathnayaka et al., (2021a) and Kondo et al., (2020), it is highly expressed at 20 DAF in parthenocarpy fruits and under drought stress conditions, and thus 100 $g \cdot m^{-2}$ P induced *FAT* when 'Takanotsume' was harvested at a more mature stage (30 DAF).

COMT showed significantly higher expression levels in both 'Takanotsume' and 'Sapporo' at 30 DAF in plants grown in 100 $g \cdot m^{-2}$ P-treated soil in the present study, but no significant expression level was observed at any harvested times for 'Shishito' and 'Sapporo' in drought stress (Rathnayaka et al., 2021a). Under parthenocarpy conditions, *COMT* is expressed at a late mature stage (50 DAF) in 'Shishito' (Kondo et al., 2020). Considering the results from the previous and present studies, the 100 $g \cdot m^{-2}$ P fertilizer treatment induces *COMT* at the early mature stage (30 DAF) in 'Takanotsume' and 'Sapporo' cultivars.

Group 4 comprised *PAL*, *4CL*, *C4H* and *C3H* and showed no significant differences in any variety at any harvest date for plants grown in soil treated with 100 and 600 $g \cdot m^{-2}$ P. *PAL*, *C4H* and *C3H* genes in plants grown under drought stress (Rathnayaka et al., 2021a) and parthenocarpy conditions (Kondo et al., 2020) did not show any high expression either, as consistent with the present study. According to Vogt (2010), the phenylpropanoid pathway is involved in the generation of flavonoids, lignin and secondary metabolites. All these group 4 genes are upstream of this pathway and thus, have little effect in inducing pungency in plants grown in 100 $g \cdot m^{-2}$ or excess P fertilizer.

The 100 $g \cdot m^{-2}$ P fertilizer contains slightly excess P compared to the standard (60 $g \cdot m^{-2}$ P) type but does not stress plants because they are grown in 100 $g \cdot m^{-2}$ P fertilizer, which provided high yields (data not shown) and did not exhibit any P toxicity symptoms. However, 600 $g \cdot m^{-2}$ P fertilizer significantly reduced harvest and capsaicinoid concentration and acted as a stress condition. Rathnayaka et al. (2021a) demonstrated that drought stress resulted in high capsaicinoid accumulation and highly expressed genes, similar to the present experimental results except for a few genes. Finally, we can conclude that groups 1, 2 and 3 genes are involved in inducing increased capsaicinoid content under non-stress conditions and without reducing the seed numbers. The P fertilizer involves not only in the capsaicinoid pathway, but also in many functions inside the chili plants. However, further studies are required to investigate the relationship between this P fertilizer and the capsaicinoid pathway.

Chapter IV

Relationship between temperature stress with taste component in chili pepper (*Capsicum* spp.)

Abstract

Capsaicinoid, sugar, and glutamic acid content of chili peppers play an important role in the food industry. According to climate change models predictions, the air temperature will increase between 1-4 °C by the end of the 21st century because of the greenhouse effect. High temperature is a stress for plants and components like capsaicinoids, sugar, and glutamic acid produced by the plant will change as a response to such stresses. Therefore, the present experiment was conducted to determine the effect of temperature stress on capsaicinoid, glucose, total sugar, Brix, and glutamic acid content in ‘Takanotsume’ (2021 and 2022), ‘Habanero’ and ‘Himo’ (2022) varieties. Experiments were conducted using two temperature treatments under greenhouse (temperature stress) and open field conditions (control) at the Education and Research Center of Alpine Field Science, Faculty of Agriculture, Shinshu University. The capsaicinoid content was measured using high-performance liquid chromatography. The glucose, total sugar and glutamic acid content were measured using a portable spectrophotometer (RQ flex). Capsaicinoid content was significantly higher in the temperature stress condition than the control condition in all varieties in both years except ‘Himo’ in 20 DAF. In both years, total sugar content, Brix percentage and glutamic acid content were significantly higher in the temperature stress condition than control condition. Only glucose content had an inverse effect, showing that the glucose content of all varieties was significantly lower in the temperature stress condition than the control condition.

Introduction

Chili peppers are widely cultivated spices around the world in the food and pharmaceutical industries (Saleh et al., 2018). Chili pepper belongs to the genus *Capsicum* in the family Solanaceae, which is the only plant genus that produces capsaicinoid (Gurung et al., 2011). Capsaicinoids are the active ingredient which gives chili peppers their hotness or burning taste (Naves et al. 2019). Capsaicinoids are produced as a result of a combination of phenylpropanoid pathways and branched-chain fatty acid pathways with the expression of *Pun1* (Aza-González et al., 2011). Apart from capsaicinoids, the flavor, aroma, nutrient content, and taste components (sugar, glutamic acid, Brix, etc.) are also important quality factors considered by the food industry (Rathnayaka et al., 2021d).

The interaction between cultivar (genetic traits) and growing environment significantly affects the accumulation of capsaicinoids in chili pepper (Topuz and Ozdemir, 2007). Drought, salinity stress (Rathnayaka et al., 2021c; 2021b), parthenocarpy (Kondo et al., 2020; 2021), light (Gangadhar et al., 2012) and fertilizer condition (Buczkowska et al., 2013) are the environmental factors which affect chili pepper pungency and fruit quality. It was also reported that, in tomato varieties, quality parameters were significantly changed at high-temperature stress (Lokesha et al., 2019).

The world is highly affected by global warming because the temperature has been increasing by 0.6 °C during the last 100 years (Root et al., 2003). In the present situation, the global temperature is continuously increasing and it was expected to increase by 0.5–2.8 °C at the end of the 21st century (Meehl et al., 2005). Plants also can have various effects due to this temperature increase, which is a stressor for plant growth and development. Components like capsaicinoids, sugar, and glutamic acid produced by the chili will also change as a response to such stresses.

There are a limited number of experiments on the effect of temperature stress on chili pepper and almost all were mainly focused only on growth and yield parameters. The relationships of environmental stresses such as drought stress, salinity stress (Rathnayaka et al., 2021c; 2021b), excess P fertilizer stress (as mentioned in Chapter II), with total sugar, glucose, Brix and glutamic acid content has been already tested, except for temperature stress. Therefore, the present experiment was conducted to determine the effect of temperature stress on capsaicinoid, total sugar, glucose, Brix, and glutamic acid content in chili pepper.

Materials and method

The experiment was conducted at the research field of the AFC, Faculty of Agriculture, Shinshu University in 2021 and 2022. ‘Takanotsume’ belonging to the *C. annuum* variety was used in 2021. ‘Takanotsume’, ‘Habanero’ (*C. chinense*) and ‘Himo’ (*C. annuum*) varieties were used for the 2022 experiment. ‘Takanotsume’ and ‘Himo’ are Japanese local chili pepper varieties, and were purchased from Takii (Kyoto, Japan). ‘Habanero’ (Tsurushin Seeds, Matsumoto) is a chili pepper originating from a local Mexican variety and is one of the most pungent chili pepper varieties in the world.

Seedlings approximately 15 cm in height were transplanted into plastic pots (18 cm in diameter, 2.2 L) filled with commercial potting medium (Nae-Ichiban; Sumitomo Forestry Landscaping Co., Ltd., Tokyo, Japan). The first set of flower buds was removed after applying the treatments. BB fertilizer 552 (N: P: K, 15:15:12) was added following the standard recommendation.

In both years, two temperature conditions were provided: a temperature stress condition (inside a greenhouse) and a control condition (outside a greenhouse). Temperature stress plants were grown in a greenhouse and were provided a 50% shade condition. Control temperature condition plants were grown outside of the greenhouse, but were also provided the 50% shade condition and shelter. An excess water condition was provided to control the effect of respectively light and drought conditions. When providing the excess water condition, overflow from the pot was retained in the plate under the pot and allowed to be absorbed through the pot base. Five plants were used for each treatment in both experiments, with one fruit from each plant harvested and used as samples for capsaicinoid analyses. Fruits bulks were collected from plants subjected to each treatment, divided into three similar samples, and used for taste component analyses in both 2021 and 2022.

Experiment 1 for capsaicinoids and taste components (conducted in 2021)

Experiment 1 was conducted from 30th June to 15th September, 2021, using the 'Takanotsume' variety and the two temperature treatments mentioned above (temperature stress – inside greenhouse and control temperature – outside greenhouse). The maximum average temperature was 42.9 °C and the minimum average temperature was 13.4 °C in the temperature stress condition (Fig. 1). In the control temperature treatment, maximum average temperature was 35.9 °C and the minimum average temperature was 11.6 °C (Fig. 1). Harvesting was done at 40 and 50 days after flowering (DAF) for capsaicinoid analysis. The number of seeds was counted before capsaicinoid analysis.

Experiment 2 for capsaicinoids and taste components (conducted in 2022)

Experiment 2 was conducted using the same temperature treatments as experiment 1 (as described above: temperature stress and control) from 15th June to 1st September, 2022. The maximum average temperature was 44.4 °C and the minimum average temperature was 14.9 °C in the temperature stress condition (Fig. 2). In the control temperature treatment, the maximum average temperature was 36.7 °C and the minimum average temperature was 13.5 °C (Fig. 2). 'Takanotsume', 'Habanero' and 'Himo' chili pepper varieties were used for experiment 2. Harvesting was done at 20 and 30 DAF for capsaicinoid analysis. The number of seeds was counted before capsaicinoid analysis.

Capsaicinoid analysis

Frozen (-80 °C) fruit samples were freeze-dried using a freeze-dryer (Islay FDU – 2000, Rikakikai Co., Ltd., Tokyo, Japan) for 24 hours, pulverized (YMB-400; Yamazen, Osaka, Japan), then 20 mL of methanol was added to the dried pepper powder and the sample was incubated at 40 °C for 1 hour (Natural Incubator, Compact NIB-10, Iwaki Glass Co., Ltd., Tokyo, Japan) to extract the capsaicinoid. The extraction sample was filtered through 125 mm filter paper (ADVANTEC®, Tokyo, Japan) and methanol was again added up to 20 mL.

Capsaicinoid was analyzed using high-performance liquid chromatography (HPLC) (YMC-Pack ODS-A, 50 × 3.0 mm, Shimadzu Corporation, Kyoto, Japan), with a column temperature of 40 °C, mobile phase of 65% methanol, a flow rate of 1 $\text{mL}\cdot\text{min}^{-1}$, and monitoring at 280 nm.

Preparation of solution to analyze glucose, total sugar and glutamic acid

Frozen chili fruit samples were ground, filtered using 125 mm filter paper (ADVANTEC®), then diluted, and glucose, total sugar and glutamic acid content were quantitatively analyzed using a portable spectrophotometer (RQ flex plus 10; Merck, Darmstadt, Germany).

Preparation of solution to analyze Brix

Extracts prepared as above were directly used to read the Brix value using a digital portable refractometer (Pen-J; ATAGO Co., Ltd., Tokyo, Japan).

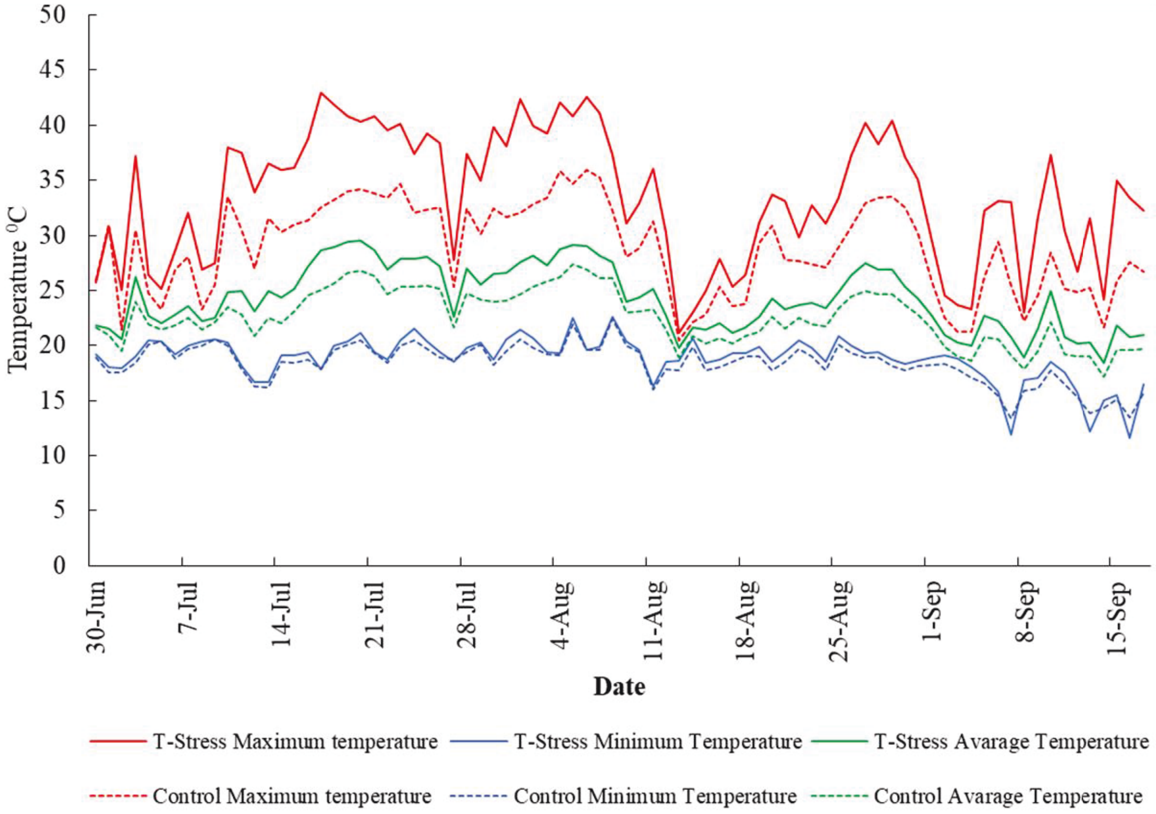


Fig. 1 Temperature changes during Experiment 1 in 2021. Red, blue and green straight lines show the temperature stress treatment maximum, minimum and average temperature, respectively. Red, blue and green dotted lines show the control temperature treatment maximum, minimum and average temperature, respectively.

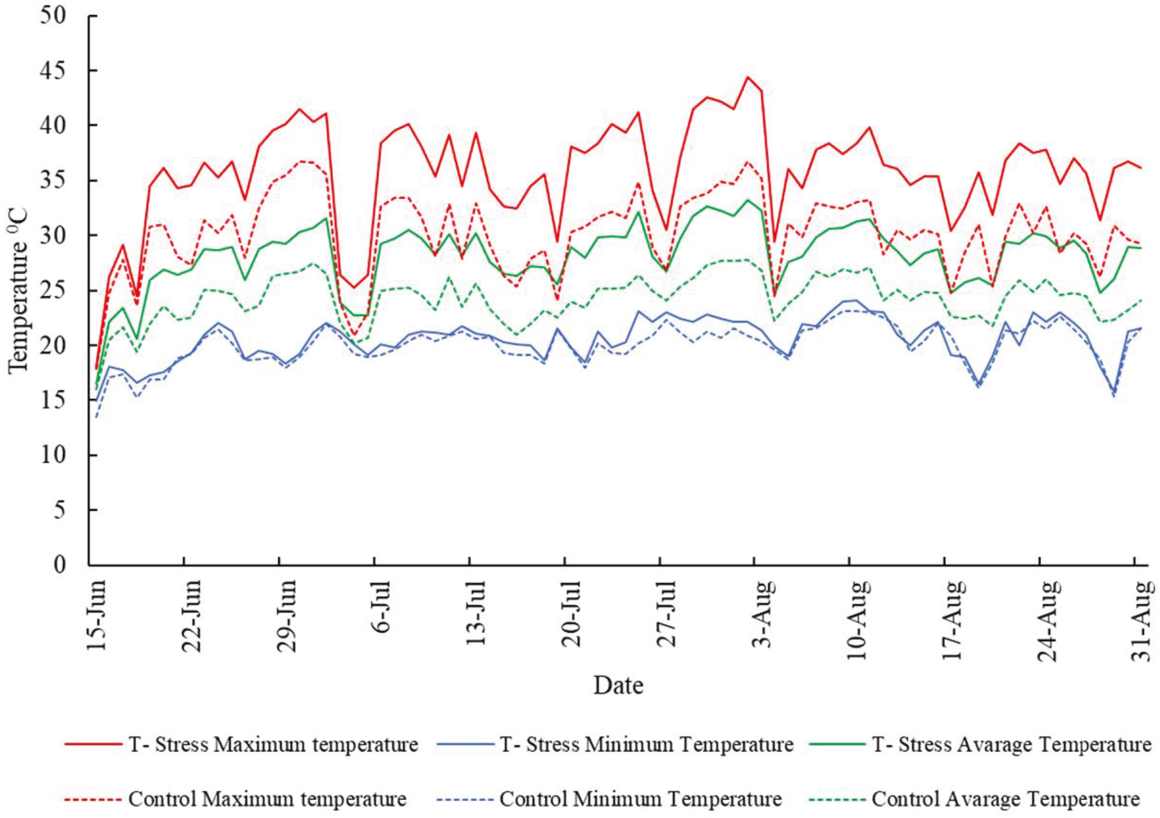


Fig. 2 Temperature changes during Experiment 1 in 2021. Red, blue and green straight lines show the temperature stress treatment maximum, minimum and average temperature, respectively. Red, blue and green dotted lines show the control temperature treatment maximum, minimum and average temperature, respectively.

Results

Average number of seeds

In Experiment 1, the average number of seeds of the ‘Takanotsume’ variety was significantly lower in the temperature stress condition than the control condition during both harvesting stages of 40 and 50 DAF in 2021 (Fig. 3).

In Experiment 2, all three varieties harvested at both 20 and 30 DAF showed a significantly lower average number of seeds in the temperature stressed condition than the control condition in 2022 (Fig. 3, 4).

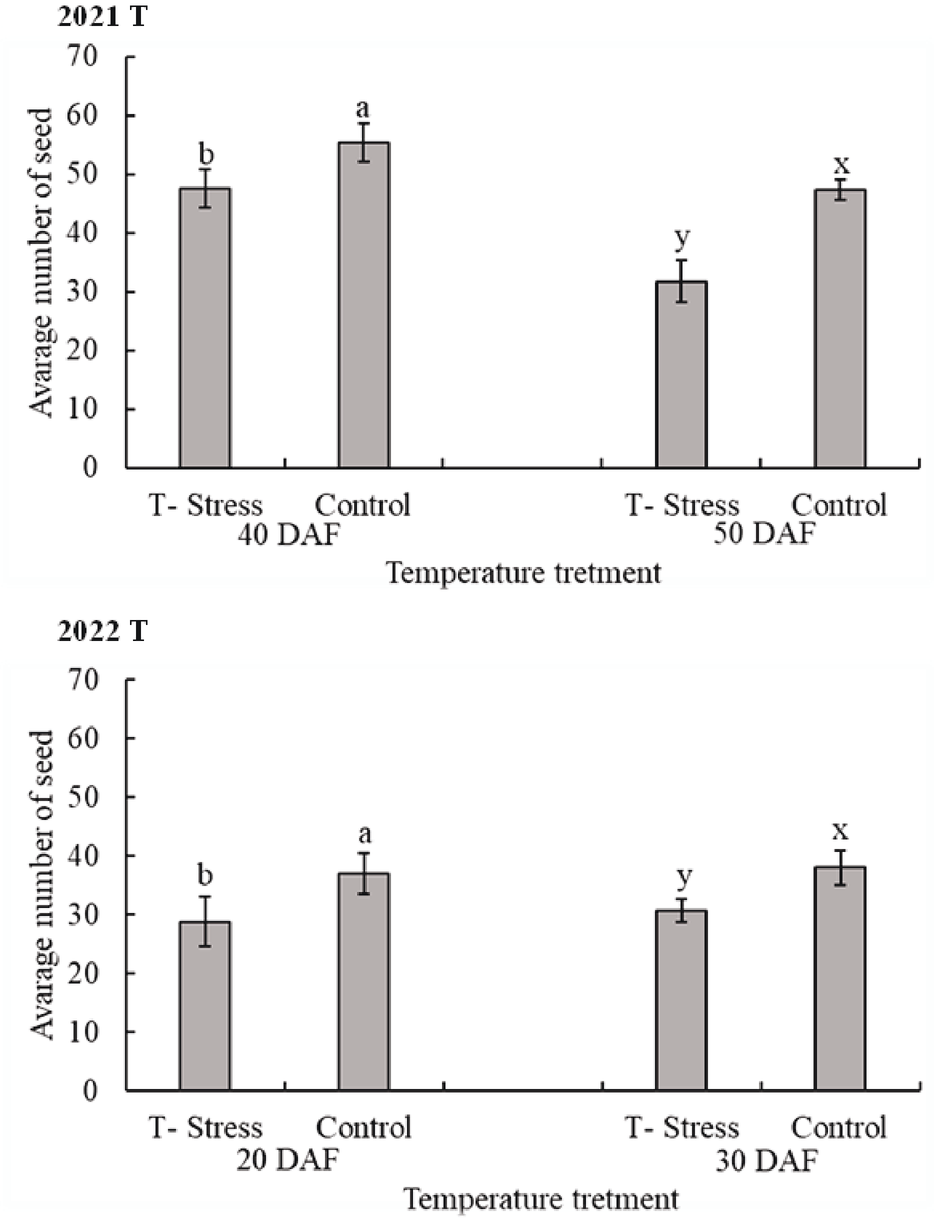


Fig. 3. Average number of seeds of ‘Takanotsume’ in 2021 (2021 T) and ‘Takanotsume’ in 2022 (2022 T) chili peppers grown in the temperature stress (T- stress) and control conditions. Lower-case letters (a) and (b) indicate significant differences between treatments for fruits at 20 and 40 days after flowering (DAF) and lower-case letter (x) and (y) indicate significant differences between treatments for fruits at 30 and 50 DAF in ‘Takanotsume’ (Tukey’s pairwise test, $p<0.05$). Error bars indicate the standard error.

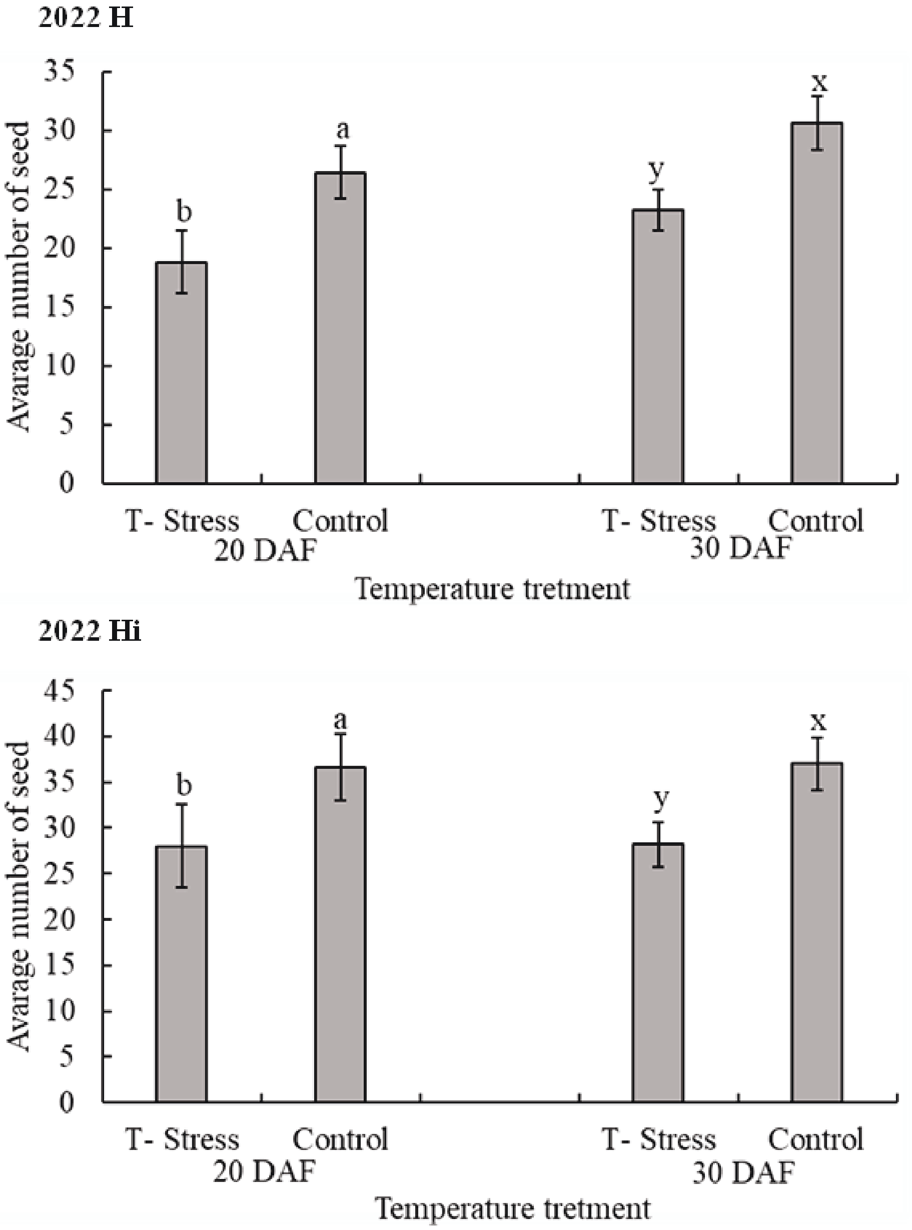


Fig. 4. Average number of seeds of ‘Habanero’ in 2022 (2022 H) and ‘Himo’ in 2022 (2022 Hi) chili peppers grown in the temperature stress (T- stress) and control conditions. Lower-case letters (a) and (b) indicate significant differences between treatments for fruits at 20 days after flowering (DAF) and lower-case letters (x) and (y) indicate significant differences between treatments for fruits at 30 DAF in ‘Habanero’ and ‘Himo’ (Tukey’s pairwise test, $p<0.05$). Error bars indicate the standard error.

Capsaicinoid content

In Experiment 1, conducted in 2021, ‘Takanotsume’ fruits harvested at both 40 and 50 DAF showed higher capsaicinoid content in the temperature stress treatment than the control treatment. (Fig. 5). However, both harvesting times (40 and 50 DAF) showed no significant difference in capsaicinoid content in each treatment.

In Experiment 2, conducted in 2022, ‘Takanotsume’ showed higher capsaicinoid content in the temperature stress compared to the control at both 20 DAF and 30 DAF (Fig. 5). ‘Habanero’ also showed higher capsaicinoid content in the temperature stress compared to the control at both 20DAF and 30 DAF (Fig. 6). However, in ‘Himo’, there was no significant difference in capsaicinoid content in between the temperature stress and the control at 20 DAF (Fig. 6), but, 30 DAF showed significantly higher capsaicinoid content in the temperature stress than the control condition in ‘Himo’.

When the capsaicinoid contents that were reported during the same treatment by the same variety were compared based on harvesting times, no significant difference was shown in ‘Takanotsume’ and ‘Habanero’. However, in ‘Himo’, fruits harvested at 30 DAF showed higher capsaicinoid content in the temperature stress condition than fruits harvested at 20 DAF in the temperature stress treatment.

Among all the treatments, the highest capsaicinoid content was reported in ‘Habanero’ in the temperature stress condition at 30 DAF, whereas the lowest was reported in ‘Himo’ at 20 DAF in the control condition.

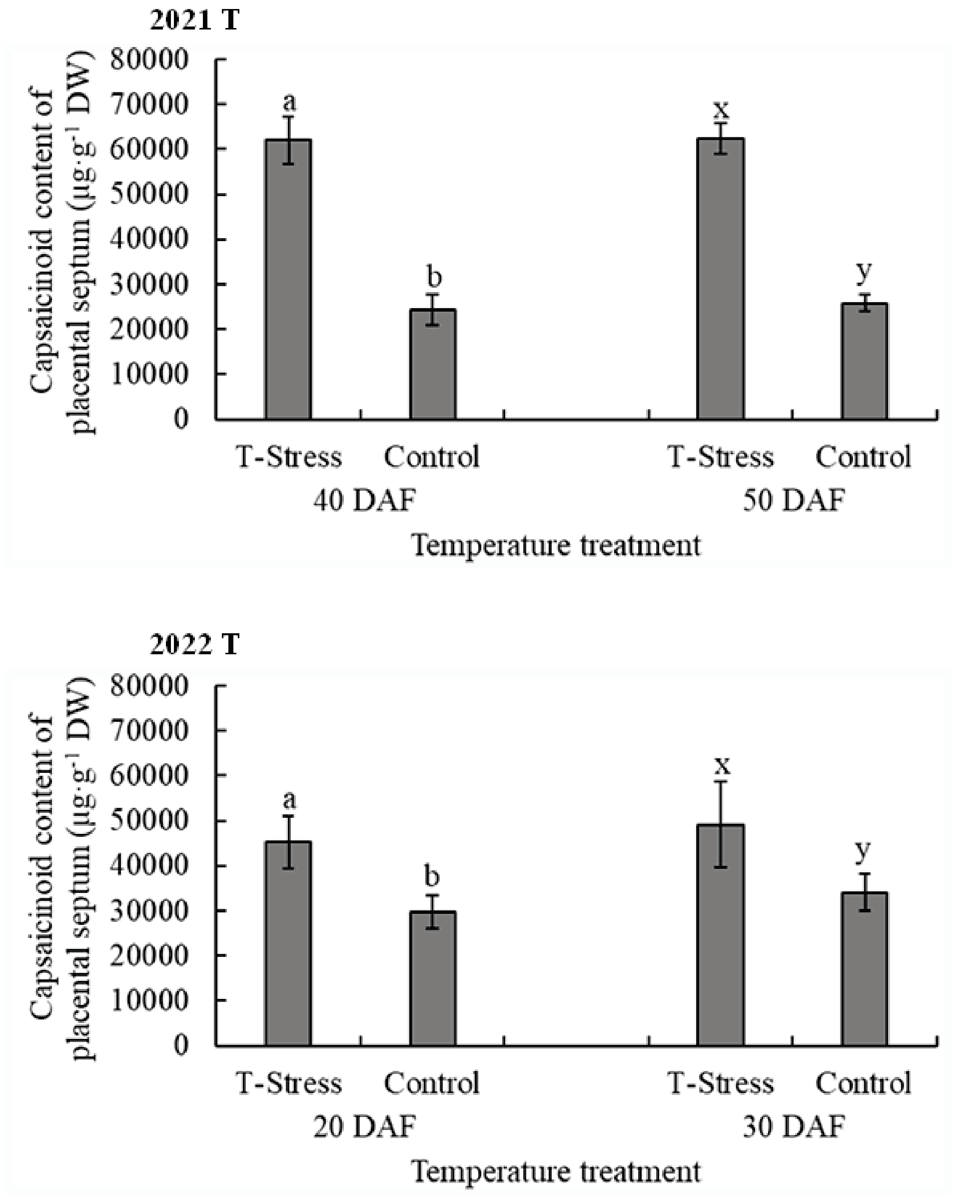


Fig. 5. Capsaicinoid content of placenta septum [μg·g-1 dry weight (DW)] in ‘Takanotsume’ in 2021 (2021 T) and ‘Takanotsume’ in 2022 (2022 T) chili peppers grown in the temperature stress (T- stress) and control conditions. Different lower-case letters (a and b) indicate significant differences between treatments for fruit harvested at 20 and 40 DAF and lower-case letters (x and y) indicate significant differences between treatments for fruits harvested at 30 and 50 DAF in ‘Takanotsume’ (Tukey’s pairwise test, $p<0.05$). Error bars indicate the standard error.

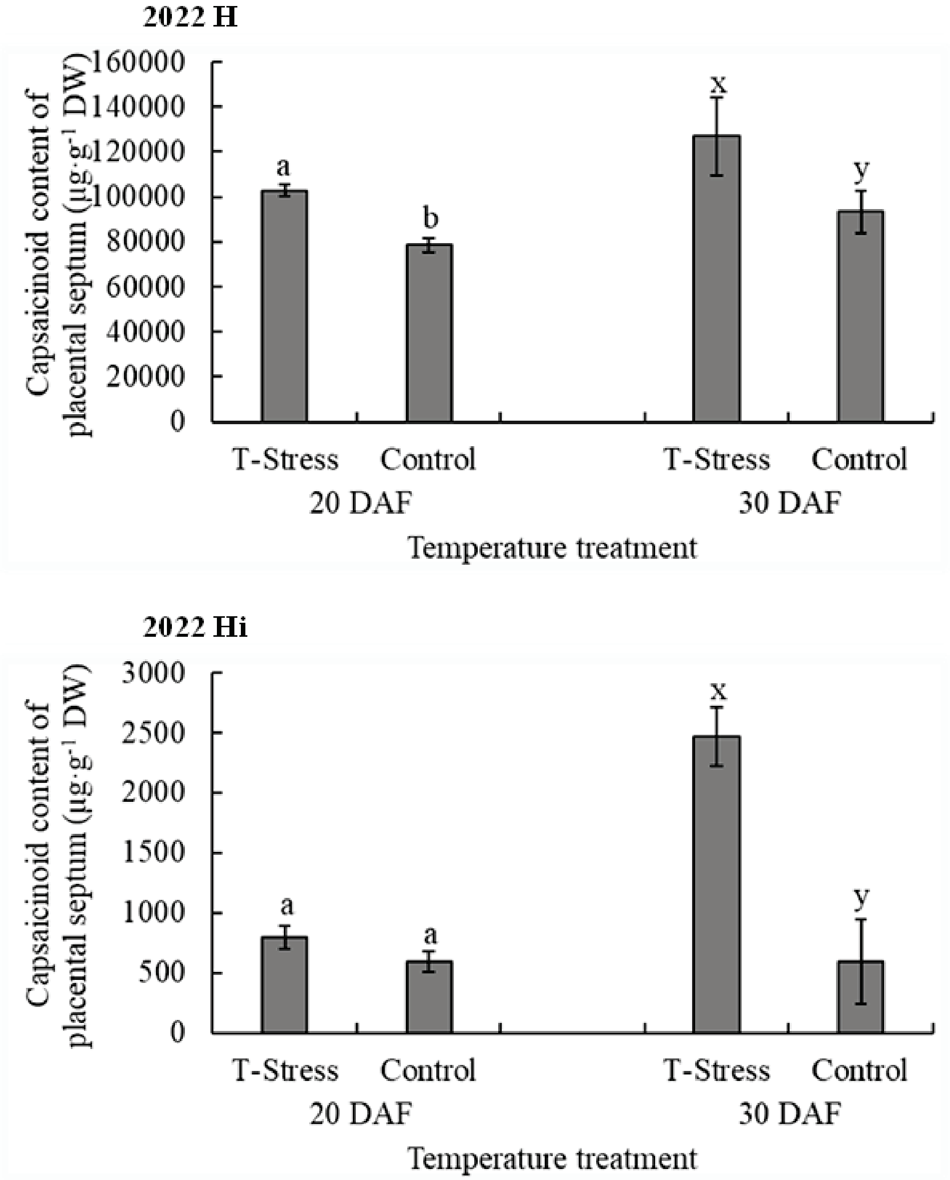


Fig. 6. Capsaicinoid content of placenta septum [μg·g-1 dry weight (DW)] in ‘Habanero’ (2022 H) and ‘Himo’ (2022 H) chili peppers grown in the temperature stress (T- stress) and control conditions in 2022. Different lower-case letters (a and b) indicate significant differences between treatments for 20 fruits and lower-case letters (x and y) indicate significant differences between treatments for 30 fruits in ‘Habanero’ and ‘Himo’ (Tukey’s pairwise test, $p<0.05$). Error bars indicate the standard error.

Glucose content

In Experiment 1 conducted during 2021, the glucose content of the ‘Takanotsume’ plants were significantly lower under the temperature stress than the control condition (Fig. 7).

In Experiment 2 conducted during 2022, the glucose content of all ‘Takanotsume’, ‘Habanero’ and ‘Himo’ varieties showed a similar pattern as in all showed less glucose content in the temperature stress condition than in the control condition (Fig. 7). Moreover, the highest glucose content was reported in ‘Himo’, while the lowest glucose content was reported in ‘Habanero’ during the temperature stress condition.

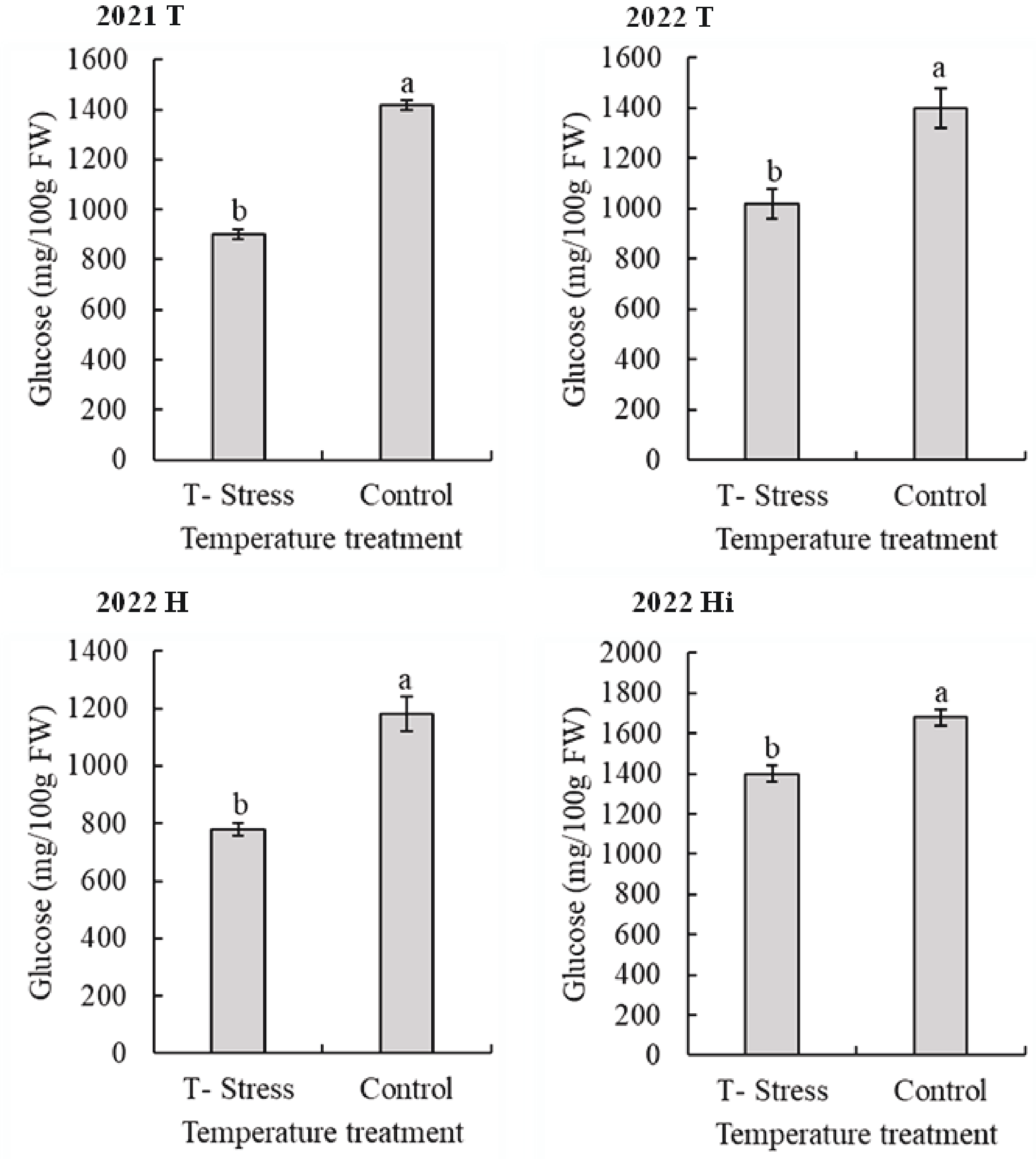


Fig. 7. Glucose content [mg/100 g fresh weight (FW)] of ‘Takanotsume’ in 2021 (2021 T), ‘Takanotsume’ in 2022 (2022 T), ‘Habanero’ (2022 H) and ‘Himo’ (2022 Hi) in 2022. Different lower-case letters (a and b) indicate significant differences between treatments (Tukey’s pairwise test, $p<0.05$) of ‘Takanotsume’, ‘Habanero’ and ‘Himo’. Error bars indicate the standard error.

Total sugar content

In Experiment 1 conducted during 2021, the total sugar content of the ‘Takanotsume’ plants was significantly higher under the temperature stress than the control condition (Fig. 8).

In Experiment 2 conducted during 2022, the total sugar content also a had similar pattern. All ‘Takanotsume’, ‘Habanero’ and ‘Himo’ varieties were higher in the temperature stress condition than in the control condition (Fig. 8). Also, as with the glucose content, the highest total sugar content was reported in ‘Himo’, and the lowest total sugar content was reported in ‘Habanero’ during the temperature stress condition.

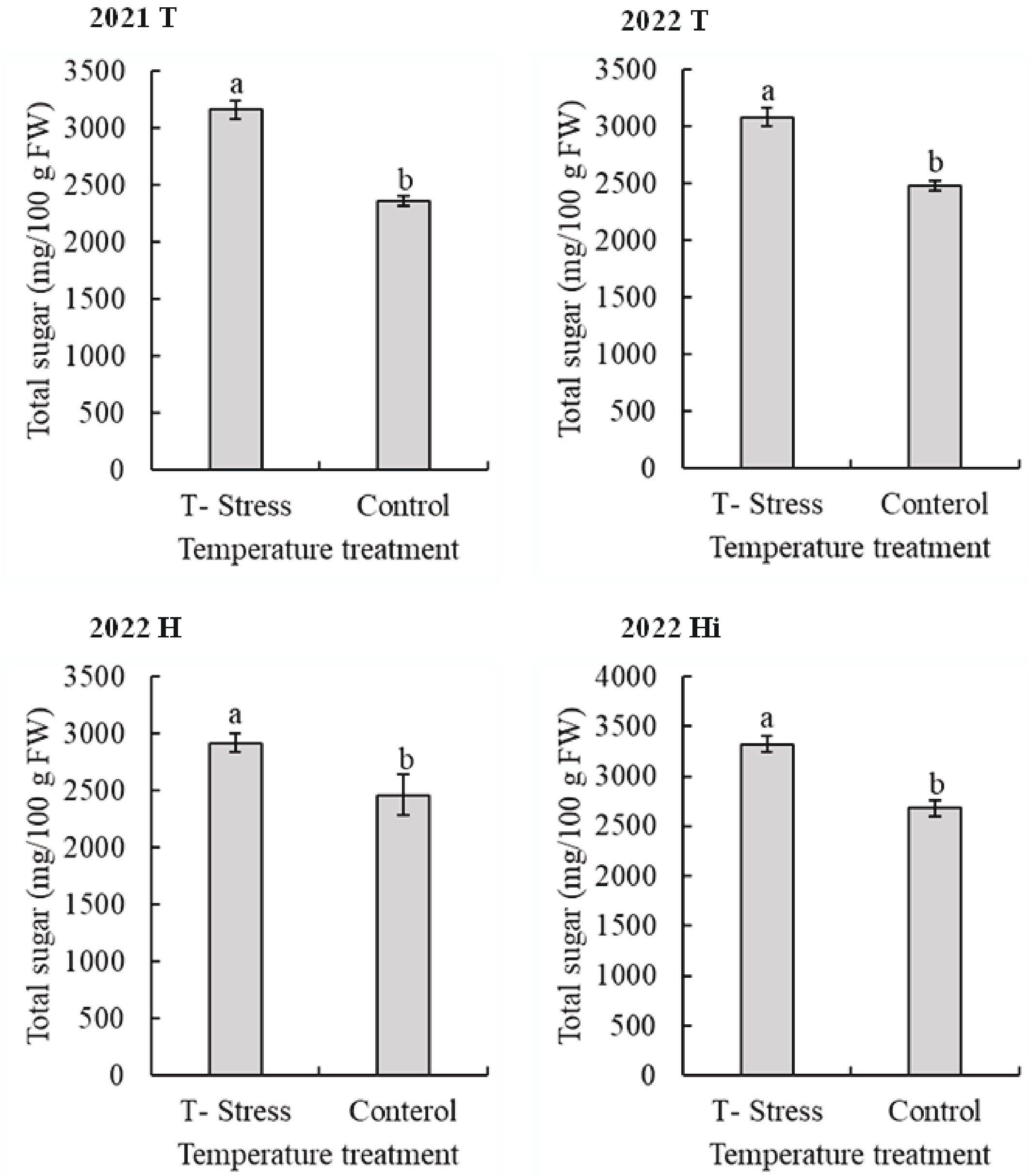


Fig. 8. Total sugar content [mg/100 g fresh weight (FW)] of 'Takanotsume' in 2021 (2021 T), 'Takanotsume' in 2022 (2022 T), 'Habanero' (2022 H) and 'Himo' (2022 Hi) in 2022. Different lower-case letters (a and b) indicate significant differences between treatments (Tukey's pairwise test, $p<0.05$) of 'Takanotsume', 'Habanero' and 'Himo'. Error bars indicate the standard error.

Brix percentage

In Experiment 1 conducted during 2021, the Brix percentage of the ‘Takanotsume’ plants was significantly higher under the temperature stress than the control condition (Fig. 9).

In Experiment 2 conducted during 2022, the Brix percentage was not significantly different between the temperature stress and control conditions in ‘Takanotsume’. However, ‘Habanero’ and ‘Himo’ showed significantly higher Brix percentage in the temperature stress condition than in the control condition (Fig. 9). Among the three varieties, the highest Brix percentage was reported in ‘Takanotsume’ and the lowest Brix percentage was reported in ‘Habanero’ during the temperature stress condition.

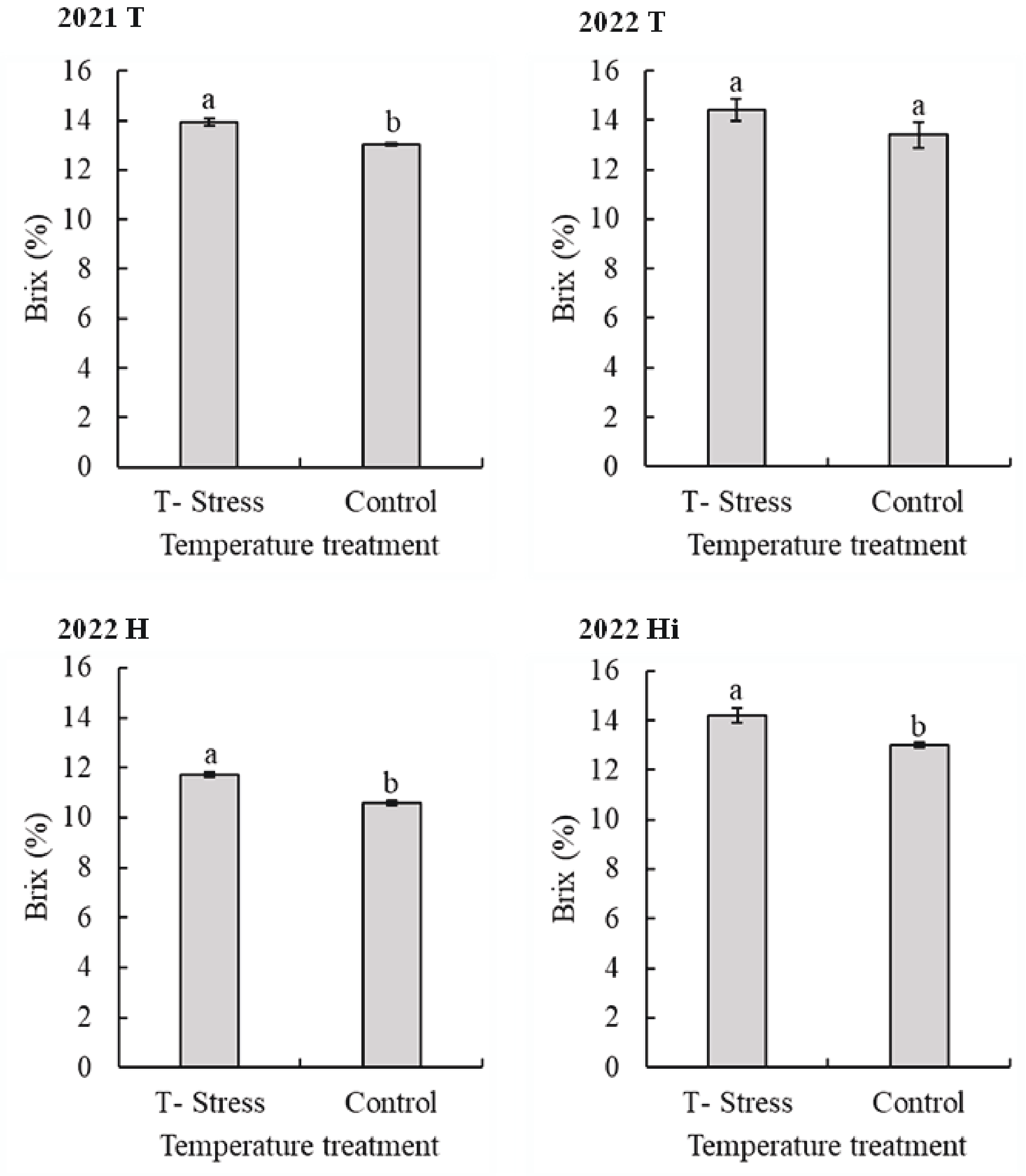


Fig. 9. Brix% of ‘Takanotsume’ in 2021 (2021 T), ‘Takanotsume’ in 2022 (2022 T), ‘Habanero’ (2022 H) and ‘Himo’ (2022 Hi) in 2022. Different lower-case letters (a and b) indicate significant differences between treatments (Tukey’s pairwise test, $p<0.05$) of ‘Takanotsume’, ‘Habanero’ and ‘Himo’. Error bars indicate the standard error.

Glutamic acid content

In Experiment 1 conducted during 2021, the glutamic acid content of the ‘Takanotsume’ plants was significantly higher under the temperature stress than the control condition (Fig. 10).

In Experiment 2 conducted during 2022, the glutamic acid content of ‘Takanotsume’, ‘Habanero’, and ‘Himo’ were also higher in the temperature stress condition than in the control condition (Fig. 10). Among the three varieties, the highest glutamic acid content was reported in ‘Takanotsume’ and the lowest glutamic acid content was reported in ‘Habanero’ during the temperature stress condition.

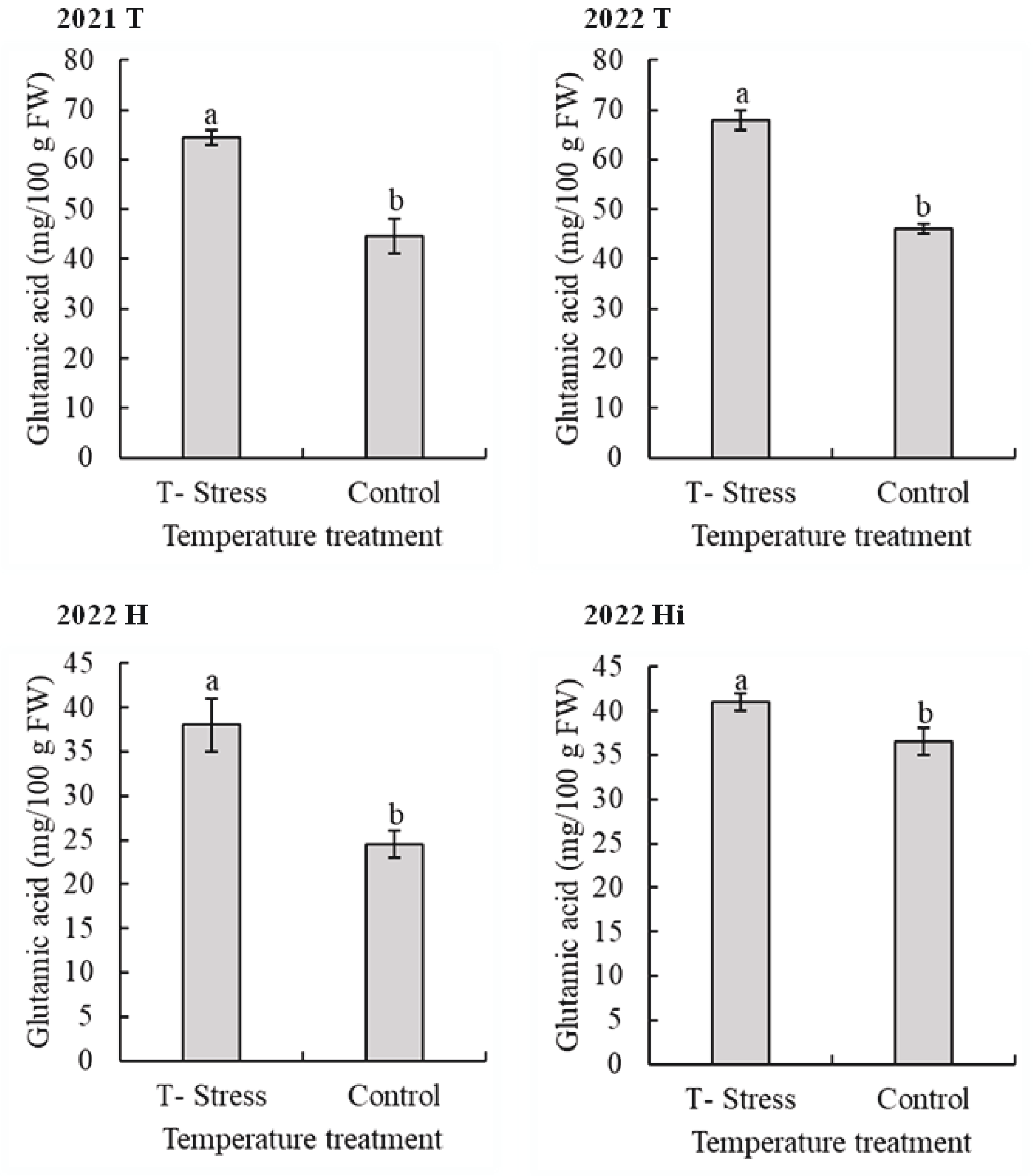


Fig. 10. Glutamic acid (mg/100 g FW) of ‘Takanotsume’ in 2021 (2021 T), ‘Takanotsume’ in 2022 (2022 T), ‘Habanero’ (2022 H) and ‘Himo’ (2022 Hi) in 2022. Different lower-case letters (a and b) indicate significant differences between treatments (Tukey’s pairwise test, $p<0.05$) of ‘Takanotsume’, ‘Habanero’ and ‘Himo’. Error bars indicate the standard error.

Discussion

An inverse relationship can be observed between the average number of seeds and the capsaicinoid content in all varieties at all harvesting stages during the both experiments conducted in 2021 and 2022. Under the temperature stress condition, capsaicinoid content was increased when the average number of seed was decreased.

In tomato plants, high temperature negatively affected the development of pollen grains, which led to poor germination of pollen and impaired pollen tube development (Raja et al., 2019). During the seed formation period of chili peppers, exposure to high temperature after anthesis severely affected development and decreased the number of seeds per fruit in ‘Shishito’ peppers (Pagamas et al., 2008). According to Kondo et al. (2020), seedless parthenocarpy fruits generated by stigma excision and 2,4-D treatment have increased pungency compared to naturally pollinated fruits that have a typical number of seeds.

The biochemical pathways of the production of lignin and capsaicin are linked where the production of one compound adversely affects the production of the other compound, resulting in a trade-off between the two compounds (Tewksbury et al., 2008). Lignin is a major structural compound which is associated with the seed coat. The need to produce lignin with the reduction of seed number becomes less, providing space to produce high capsaicinoid in a trade-off scenario. This phenomenon could be possible and is the most suitable explanation for the higher amount of capsaicinoid content reported during the temperature stress condition in the present study.

However, the ‘Himo’ variety was an exception with regards to the capsaicinoid content reported during the stress conditions. In the case of ‘Himo’ pepper, HPLC could not detect a sufficient amount of capsaicinoid at 20 DAF to support the above results.

Apart from that, in drought stress conditions, capsaicinoid content has been increased with maturity (20 DAF to 50 DAF) and the highest capsaicinoid content was reported in a drought stress condition rather than standard and excess water conditions (Rathnayaka et al., 2021c). Also, according to the Rathnayaka et al. (2021b), capsaicinoid content was increased in delayed harvesting and with increasing soil salinity. However, the present study showed no significant difference between 40 and 50 DAF or between 20 and 30 DAF in both the temperature stress and control treatments in 2021 and 2022 respectively. Rathnayaka et al. (2021b) measured the capsaicinoid content after a 30 days gap altogether, but in the present study, the gap of harvesting was 10 days which was clearly not sufficient to show a significant difference in the capsaicinoid content.

Temperature stress is also an environmental stress like drought and salinity stress; therefore, in the temperature stress condition also, the capsaicinoid content was significantly higher as in the abovementioned drought and salinity stress experiments. Harvell and Bosland (1997) also reported that pungency is related to environmental conditions, and that it increases in response to stressful conditions.

According to the Rathnayaka et al. (2021c), glucose content was significantly low in the drought stress condition compared to the excess water condition in most of the harvesting stages of some chili pepper varieties. Also, in the excessive salinity stress condition, the glucose content of some chili varieties significantly decreased with delayed harvesting compared to the control condition (Rathnayaka et al., 2021b). In the present experiment also, the results of the temperature stress treatments of both years showed a similar tendency of producing less glucose than the control treatment in all varieties.

In both drought and salinity stress conditions, total sugar content and Brix percentage of some chili varieties which were harvested at the late stage tended to increase compared to the control conditions (Rathnayaka et al., 2021b; 2021c). Supporting Rathnayaka et al., temperature stress, which is also an environmental stress to plants, stimulated an increase in the total sugar content and Brix percentage compared to the control.

According to the tomato experiment done by Shivashankara et al. (2014), TSS (Total soluble solid /Brix percentage) increased in all used genotypes under temperature stress compared to the control, which also supported our present experiment. However, in another tomato experiment, the total sugar content decreased significantly in all the genotypes under high temperature stress conditions compared to control (Lokesha et al., 2019). Gautier (2005) also reported that total sugar content was reduced in cherry tomatoes when temperature was increased. According to the Fleisher et al. (2006), temperature stress affected fruit maturity and growth through influencing acid invertase and sucrose synthase enzyme regulation and also regulation of sugar transport into the tomato fruits. It is also reported that different plant species have developed different coping mechanisms against different stress conditions during the history of their evolution (Chen and Murata, 2002). Because of this, chili and tomato, which are two different species, might have different strategies to survive the same stressful situations. This might be a possible reason for less total sugar production by tomato and high total sugar production by chili during temperature stress.

Considering the glutamic acid content measured in the present study, a higher amount was reported during the temperature stress. This also agrees with the results of Rathnayaka et al. (2021b; 2021c) where in the majority of chili pepper cultivars used, glutamic acid content showed a higher level in both drought and excessive salinity stress treatments compared to the control treatments.

According to the Umami Information Center (<https://www.umamiinfo.com/richfood/foodstuff/tomato.html>, December 22, 2022), tomato fruit contains high contents of the umami provider glutamic acid, especially as the fruit ripens. However, the susceptibility of plants to high temperature differs according to genotype and developmental stage (Wahid et al., 2007).

In conclusion, the pungency was enhanced when temperature stress was applied. Considering the total sugar content and the Brix percentage, the sweetness of chili fruit also could be increased with temperature stress. Therefore, if farmers use temperature stress conditions rather than growing plants at the normal environmental temperature, they can get more pungent, sweet and umami tasting chili fruits.

Chapter V

General Discussion

Plants face different environmental conditions, which could be favorable or unfavorable, during their lifetime (Liu et al., 2017). These changes in environmental factors influence the growth and development of the plants. When these environmental factors affect the plants adversely, they are considered a stress to the plants (Verma et al., 2013). Most importantly, these stress conditions are major decisive factors of crop yield and quality in agriculture. Abiotic stress such as drought stress, salinity stress, light stress, heavy metal stress, nutrients stress, and temperature stress could drastically affect the agriculture industry (Mareri et al., 2022). Chili is a major agricultural crop which is distinguished by its pungency. Capsaicinoid determines the pungency of chili fruits and the amount of capsaicinoid varies with genotype and the environmental stress faced by the chili cultivations (Gurung et al., 2011). Apart from capsaicinoid, glucose, total sugar, Brix percentage, and glutamic acid content also determine the chili fruit quality and are also affected by the previously mentioned stress conditions (Iwai et al., 1979; Rathnayaka et al., 2021b).

When plants are grown in excess fertilizer, it is also a kind of stress to the plants. Application of fertilizers beyond the recommended level without soil testing can lead to implications such as soil degradation, nutrient imbalance, soil structure destruction, bulk density increase (Sayci, 2012). According to Mishima et al. (2010), most agricultural lands in Japan consist of high P fertilizer levels due to the over application of P fertilizer. Due to global warming, the global temperature is increasing continuously (Meehl et al., 2005) and this also affects plant growth and development, including the chili.

Excess P and high temperatures can affect the chili fruit quality by changing capsaicinoid, glucose, total sugar, Brix percentage, and glutamic acid content. Therefore, it is necessary to reveal the relationship between P fertilizer and high temperature stress with capsaicinoid, sugar, total sugar content and Brix value in chili peppers.

In Chapter II, the effect of P fertilizer level on taste components of the chili pepper was investigated. The results revealed that capsaicinoid content was increased when the amount of P fertilizer was increased. However, capsaicinoid content was decreased when excess P fertilizer (300 $g \cdot m^{-2}$) was applied. Glucose, total sugar and Brix were increased in excess P fertilizer application treatments. Agreeing with our results, it is also reported that adding high P fertilizer to tomato plants increased the yield and total soluble solid content (sugar, amino acids, and organic acids) (Filho et al., 2020). However, lycopene content of tomato reduced with the addition of excess P fertilizer (Liu et al., 2011).

The pungency of chili comes from the capsaicinoid; hence, pungency increases when P fertilizer is applied to the appropriate level but is weakened when it is over-applied. Larger amounts of P fertilizer may increase sweetness but not when it is over-applied. Therefore, it is vital to measure the soil-available P level before planting chili pepper to avoid adding excess P fertilizer and to obtain good quality chili fruits.

During the P experiment Chapter II, the highest amount of capsaicinoid was reported at 100 and 200 $g \cdot m^{-2}$ P and at excess P (300 $g \cdot m^{-2}$), the capsaicinoid decreased. Assuming that this may be due to the differences in capsaicinoid synthesis, we wanted to check the expression levels of 18 genes in the capsaicinoid biosynthesis pathway. Therefore, the experiment in Chapter III was conducted to find out the relative expression levels in the capsaicinoid biosynthesis genes along with the capsaicinoid content.

According to the results, capsaicinoid content of both ‘Takanotsume’ and ‘Sapporo’ were higher in 100 $g \cdot m^{-2}$ P fertilizer treatment than in 600 $g \cdot m^{-2}$ P (excess P) treatment. We could observe differences in expression levels of different genes among the 18 genes of ‘Takanotsume’ and ‘Sapporo’ varieties depending on the variety and harvesting stage. Most of the genes that were significantly highly expressed during the 100 $g \cdot m^{-2}$ P fertilizer treatment belonged to the branched-chain fatty acid pathway. Interestingly, most genes belonging to the upper part of phenylpropanoid pathway did not show a significant difference in gene expression either in 100 $g \cdot m^{-2}$ P or in 600 $g \cdot m^{-2}$ P fertilizer treatments.

In Chapter IV, the effect of temperature stress on the taste components of the chili pepper was investigated. Temperature stress given in the greenhouse contributed to an increase in capsaicinoid, total sugar, glutamic acid content, and brix percentage, except for glucose content. Our results in the temperature stress condition agree with the experiment conducted by Rathnayaka et al. (2021c; 2021b) on drought and salinity stress towards the taste components of chili plants. Similar to high temperature, extreme drought and salinity conditions are also stress situations for chili plants and they may have the same defense mechanisms by increasing capsaicinoid content and other taste components except for glucose.

Lignin is a structural compound in the seed coat and it is also produced in part of a capsaicinoid biosynthesis pathway (phenylpropanoid pathway). There is a trade-off between lignin and capsaicinoid in the capsaicinoid biosynthesis pathway (Tewksbury et al., 2008). During salinity and drought stress (Rathnayaka et al., 2021a; 2021b) and also during a parthenocarpy experiment (Kondo et al., 2020), as a result of a decrease in the number of seeds, capsaicinoid content was increased. This also confirms the trade-off between lignin and capsaicinoid. The temperature experiment in Chapter IV also showed an inverse relationship between capsaicinoid content and average number of seeds. That means capsaicinoid content is high during high temperature stress conditions with a low average number of seeds. High temperature stress affects loss of pollen viability of the chili pant (Shaked et al., 2004). As a result of that, a high temperature stress condition leads to a reduced seed number. Then, the capsaicinoid content becomes high because of the trade-off. Therefore, it is clear that temperature stress does not directly affect capsaicinoid biosynthesis, but it indirectly affects the increase in capsaicinoid.

In contrast to the Chapter IV P experiment, the average number of seeds did not increase or decrease with respect to the 100 $g{\cdot}m^{-2}$ P fertilizer level or 600 $g{\cdot}m^{-2}$ P fertilizer level in the Chapter III experiment. However, capsaicinoid content decreased at the 600 $g{\cdot}m^{-2}$ P fertilizer level (excess P) more than at the 100 $g{\cdot}m^{-2}$ P fertilizer level. That means the capsaicinoid content was reduced not because of the trade-off with lignin, but because the capsaicinoid synthesis ability of the placental septum was reduced with excess P. Therefore, high temperature stress reduces the seed number and increases the capsaicinoid content in chili pepper fruit. On the other hand, phosphate fertilizer application also similarly affects the capsaicinoid content in the fruit, but does not change the seed number.

However, Kondo et al. (2021) mentioned that 'Shishito' fruits with few seeds are not always highly pungent and that fruits with few seeds had a larger fluctuation of capsaicinoid content than fruit with many seeds. They also stated that the upstream genes of the phenylpropanoid pathway (*PAL*, *C4H*, *4CL*, *C3H*, *HCT* and *COMT*) did not show any significant correlations with capsaicinoid concentration and relative gene expression level in 'Shishito' fruits (Kondo et al., 2021). Rathnayaka et al. (2021a) also reported that the upstream genes of the phenylpropanoid pathway (*PAL*, *C3H*, *HCT*, *C4H*, and *COMT*) did not show a significant difference in gene expression levels under drought stress and excess water stress. In Chapter III, the present study also showed that the chili fruits planted in 100 g·m^{-2} P or 600 g·m^{-2} fertilizer levels did not show significant differences in the relative expression levels of most upstream genes of the phenylpropanoid pathway (*PAL*, *4CL*, *C4H* and *C3H*) in any variety at any harvesting stage. Considering all of the results obtained by the three studies above, expression of the upstream genes of the phenylpropanoid pathway does not appear to have a significant relationship with fluctuation of capsaicinoid content by parthenocarpy, drought stress and excess P fertilizer. The upstream genes of the phenylpropanoid pathway are also involved in the production of lignin which is the main structural component of the seed coat (Tewksbury et al., 2008). Therefore, the absence of a direct relationship between the number of seeds and capsaicinoid content is possible. Overall, the present study revealed that the P level and temperature stress respectively, directly and indirectly affect taste components including capsaicinoid. Continuous addition of P fertilizer to the cultivation fields does not produce quality chili fruits. Instead, farmers could measure the soil available P to avoid adding extra P and this would also be cost effective. At the same time, for the farmers who want to produce more pungent chili, this will prevent addition of excess P to the fields, which reduces the pungency.

When considering the high temperature stress over normal environmental temperature, the pungency increases. Based on our results, we can say that farmers can produce highly pungent, quality chili fruits if they provide temperature stress to the chili plants, but we cannot tell the exact optimum temperature to produce the optimum quality. Therefore, more research has to be done to investigate the optimum temperature for chili cultivation.

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