Doctoral Dissertation (Shinshu University)

Effect of environmental stresses on chemical components related to taste, growth and yield of chili pepper (*Capsicum* spp.)

(環境ストレスがトウガラシ（*Capsicum* spp.）の呈味成分含量，成長および収量に及ぼす影響)

March 2021

R. M. Sangeeth M. B. Rathnayaka

Table of Contents

Chapter I

General Introduction

The chili pepper (*Capsicum annuum)* is the fruit of plants from the genus *Capsicum*, member of the nightshade family, Solanaceae, are widely grown for their fruits, which may be eaten fresh or cooked (e.g., salads, baked dishes, salsa, pizzas, etc.), used as a dried powder, or processed into oleoresins. It has been domesticated for more than 6000 years (Perry et al., 2007). World production of hot peppers is mostly in tropical countries, such as India, Indonesia, Myanmar, Bangladesh, Sri Lanka, Pakistan, and Thailand (FAOSTAT, 2014). Peppers are commonly broken down into three groups: bell peppers, sweet peppers, and hot peppers. Most popular pepper varieties fall into one of these categories or as a cross between them (Channabasavann and Setty, 2000).

Capsaicinoid and sugar contents in chili pepper are two important factors in the food industry, and pungency comes from a group of compounds called capsaicinoids. Variation in pungency can be attributed to cultivar differences; additionally, hereditary and environmental factors also influence the presence of capsaicinoids in pepper (Zewdie and Bosland, 2000). Also, capsaicinoid accumulation is related to a fruit's age, size, and stage of development (Estrada et al., 1997). The quantity of capsaicin varies by variety, with growing conditions and exposure to stresses causing production of stronger pungent pepper pods. For an example, when a Habanero plant was stressed (low water), the concentration of capsaicin increased in some parts of the fruit (Ruiz-Lau et al., 2011). Similarly, capsaicinoid content is increased under drought stress (Estrada et al., 1999) with some of the increase resulting from varietal differences (Sung et al., 2005). In addition, the stress level and physiological responses related to growth, yield and pungency level were good criteria for evaluating hot pepper cultivars with high capsaicinoid levels under drought stress (Sung et al., 2005).

In the case of tomatoes, water stress affects the sweetness of the fruit as a result of increases in the glucose and sucrose contents, and improves fruit quality via an increase in the concentrations of important acids. Environmental factors, such as temperature, solar radiation, water stress, and soil nutrient concentrations, influence sugar and glutamic acid contents in tomato fruit (Ortiz et al., 2007). Saito et al. (2006) reported that salinity stress can positively influence the taste component in tomato fruits. More recent research has focused on salt tolerance among food crop tomatoes (Li et al., 2011), and examined the effects of soil salinity on halophytic plant species (Ungar, 1991).

However, high concentrations of salts in soil and drought stress conditions are responsible for large decreases in the yield of a variety of crops worldwide. It was estimated that about 20% (45 million ha) of irrigated land, producing 1/3 of the world's food, is salt-affected (Shrivastava and Kumar, 2015). Most of the plant species fell into a stressed condition because of the high salinity levels in the soil. Environmental stresses, such as temperature, light, water stress, soil nutrients, salinity levels in the soil or fertilizer stress, can affect pungency and the other taste components in chili pepper. As mentioned, taste components in chili pepper are very important factors in the global food industry. Specific pungency levels must be maintained for food manufacturers to label products as mild, medium, or hot. Therefore, it is very important to reveal the behavior of taste components and plant physiological parameters of the chili pepper plants under stress conditions.

To the best of my knowledge, few investigations regarding the influence of environmental factors, especially water supply and soil salinity, on the pungency and contents of sugar and glutamic acid in chili pepper fruit have been undertaken. Therefore, the present study investigated the taste components of chili pepper under environmental stress. Accordingly, this study was composed of the following experiments: in Chapter II, an experiment was conducted to determine the relationship between water supply and contents of sugar and capsaicinoids in fruit of chili pepper (*Capsicum annuum* L.). In Chapter III, an experiment was conducted to determine the relationship between salinity stress and the contents of sugar, glutamic acid and capsaicinoids in the fruit of chili peppers (*Capsicum* spp.) was conducted. In Chapter IV, an experiment was conducted to determine the relationship between the soluble solids metric brix and other traits in capsicum peppers. In Chapter V, an experiment was conducted to determine drought stress-induced increase of pungency and expression of capsaicinoid biosynthesis genes in chili pepper (*Capsicum annuum* L.).

Chapter II

Relationship between Water Supply and Contents of Sugar and Capsaicinoids in Fruit of Chili Pepper (*Capsicum annuum* L.)

Abstract

Environmental factors influence the contents of taste components, such as capsaicinoid compounds, in the fruit of chili pepper (*Capsicum annuum*). The present research was conducted to evaluate the effect of water supply and harvesting date after flowering on sugar and capsaicinoid contents in fruit of the Japanese chili pepper cultivars ‘Botankosho’, ‘Fushimiamanaga’, ‘Manganji’, and ‘Sapporo Oonaga Nanban’. The experiment was conducted in a greenhouse from April to October in 2016 and 2017. Three water supply treatments were applied: 50 mL (drought), 130 mL (standard), and 260 mL (excess) per application. Fruit were harvested at 20, 30, 40, and 50 days after flowering (DAF). The contents of glucose, glutamic acid, and total sugar were measured using a portable spectrophotometer, and capsaicinoid content was measured by HPLC. Total sugar content and Brix tended to increase with delay in harvesting, whereas glucose content did not change significantly with number of DAF. Sugars in the fruit were dominated by fructose, and the ratio of fructose content to total sugar content increased as the fruit matured. Glutamic acid content in the fruit increased up to 40 DAF, and thereafter remained unchanged or decreased. The capsaicinoid content of the fruit increased with fruit maturation. Elevation in water supply induced an increase in the fruit glucose content and decrease in total sugar and glutamic acid contents. Previous studies of tomato (*Solanum lycopersicum* L.) have shown that total sugar and glucose contents decrease in response to increase in water supply. Therefore, it is speculated that sugar

metabolism and accumulation differ in the fruits of tomato and chili pepper. The highest capsaicinoid content in chili pepper fruit was observed in response to the drought treatment.

Introduction

Chili pepper (*Capsicum annuum* L.) is a species in the genus *Capsicum*, which is classified in the Solanaceae. Chili pepper is widely grown for the fruit, which may be eaten as a fresh vegetable or used in the form of dried powder as a spice. Chili pepper originated in Mexico (Kraft et al., 2014) and was domesticated more than 6000 years ago (Perry et al., 2007). Following the Columbian Exchange, many cultivars of chili pepper were disseminated throughout the world.

The pungency of chili peppers is derived from a group of heat-producing alkaloids, including capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) and several related compounds, which are collectively termed capsaicinoids (Kosuge and Inagaki, 1962; Suzuki and Iwai, 1984). The greater the concentration of capsaicin, the greater the pungency of the fruit. Generally, capsaicin and dihydrocapsaicin are responsible for 90% of the fruit pungency (Madhumathy et al., 2007). The amount of capsaicin in a chili pepper fruit also varies according to genetic and environmental factors. Capsaicinoid accumulation is associated with the age, size, and developmental stage of the fruit (Estrada et al., 1997; Zewdie and Bosland, 2000). Water-stressed chili pepper plants usually produce fruit with greater pungency. In water-stressed plants of *C. chinense* Jacq., the concentration of capsaicin increases in some parts of the fruit (Ruiz-Lau et al., 2011). However, in the case of *C. annuum*, a similarly detailed analysis has not been conducted.

Water stress controls the physiological processes that determine the quality and quantity of growth (Kramer, 1969). According to Ullah et al. (1993, 1994), certain environmental stresses result in significant increases in the contents of glucose and fructose, and in some cases sucrose, ascorbic acid, and citric acid, in fruit of faba bean (*Vicia faba* L.) and tomato (*Solanum lycopersicum* L.). Moreover, water stress affects the sweetness of tomato fruit as a result of increase in the glucose and sucrose contents, and improves fruit quality through increase in the concentrations of important acids. Environmental factors, such as temperature, solar radiation, water stress, and soil nutrient concentrations, influence sugar and glutamic acid contents in tomato fruit (Ortiz et al., 2007). However, to the best of my knowledge, few investigations of the influence of environmental factors, especially water supply, on the pungency and contents of sugar and glutamic acid in chili pepper fruit have been undertaken. Therefore, it is necessary to determine the relationship between water stress and contents of taste components (sugars, glutamic acid, and capsaicinoids) in chili pepper fruit to enable production of fruit with stable sweetness and pungency. The present study was conducted to determine the contents of sweetness components and capsaicinoids in response to three water supply treatments.

Materials and Methods

Plant materials and experimental design

From April to October 2016 an experiment was conducted in a greenhouse at the experimental farm (733 m a.s.l) of the Education and Research Center of Alpine Field Science, Faculty of Agriculture, Shinshu University in Minamiminowa, Nagano, Japan. The experiment was repeated in the same location in March to October 2017. In 2016, two Japanese cultivars of chili pepper, ‘Manganji’ and ‘Fushimiamanaga’, were used in the experiment. Both cultivars are non-pungent vegetable cultivars that originated in Kyoto Prefecture. Seeds of 'Manganji' were purchased from Noguchi Seeds (Saitama, Japan) and seeds of ‘Fushimiamanaga’ were purchased from Takii Seed Co. Ltd. (Kyoto, Japan). In addition, the local pungent 'Botankosho', which is used as a traditional vegetable in northern Nagano Prefecture, was used from seeds donated by the Madarao Botankosho Conservation Society (Nakano, Nagano, Japan). In 2017, I included an additional pungent cultivar, ‘Sapporo Oonaga Nanban’ (Tsurushin Seed, Matsumoto, Japan; hereafter ‘Sapporo’), which is a local cultivar that originated in Hokkaido (Fig. 1). Seeds were sown on 29 April 2016 and 22 March 2017. The seedlings were grown in a greenhouse in 75 mm plastic pots filled with a commercial potting medium (Nae-ichiban, Sumitomo Forestry Landscaping Co., Ltd, Tokyo, Japan). During the seedling rearing period, the greenhouse was heated by using oil heaters at night until early April to keep above 15 °C in minimum temperature.

Fig. 1. Fruit of Japanese chili pepper (*Capsicum annuum*) cultivars included in the study: (a) ‘Manganji’ (Noguchi), (b) ‘Fushimiamanaga’ (Takii), (c) ‘Botankosho’ (Local) and (d) ‘Sapporo Oonaga Nanban’ (Tsurushin).

Seedlings approximately 150 mm in height were transplanted to plastic pots (diameter 18 cm, volume 1.8 L) filled with 1 kg of the same commercial potting medium on 6 June 2016 and 8 June 2017. In both years, the stress treatment was applied starting one week after the seedlings were transplanted. All flowers that had flowered before the stress treatment was applied were removed.

During the cultivation period a single application of slow-acting home gardening fertilizer (N:P:K, 10:10:10; Shizen Oyokagaku Co. Ltd, Nagoya, Aichi, Japan) was applied (on 16 August 2016 and 5 August 2017). The plastic pots filled with 1 kg potting medium were filled to the brim with 130 mL water. Therefore, as treatments, applied three volumes of water in individual applications to represent drought (D; 50 mL water per application), standard water supply (S; 130 mL water per application), and excess water supply (E; 260 mL water per application). In the excess water supply treatment, the excess water overflowing from the pot was retained in a dish placed under the pot and allowed to be absorbed through the pot base. The water supply treatments were applied with consideration of the daily temperature and weather. On sunny days and/or when the day temperature exceeded 30 °C, water was applied three times per day. On rainy or cloudy days and/or when the day temperature was less than 30 °C, water was applied twice per day. During the time period of experiment average temperature of the green house was 27.8 °C (Maximum 35.2 °C, Minimum 20.5 °C) in 2016 and 29.4 °C (Maximum 35.6 °C, Minimum 22.1 °C) in 2017. Throughout the experiment, other management practices were applied equally to all plants in the same greenhouse.

Fruit harvested for each water supply treatment and at each harvesting date were used for analysis of the fruit weight, content of glucose, total sugar (glucose and fructose), glutamic acid content, Brix percentage, and capsaicinoids content. Six individuals of each cultivar were used for each treatment. Fruit from each individual were harvested at 20, 30, 40, and 50 days after flowering (DAF). During the stress treatment, each flower was tagged at the end of anthesis to ensure that fruit could be harvested at 20, 30, 40, and 50 days after flowering. The harvested fruit were stored at -80 °C until analysis. Three samples each comprising 50 g of fruit (approximately 1 - 6 fruit) were chosen randomly for analysis of taste components. The sampling level reflected that the extract from 50 g of fruit tissue was required for spectrophotometric analysis. A randomized complete block design was used for the experiments.

In 2018, a third experiment was conducted to determine capsaicinoid production in the placenta. Capsaicinoids in the placenta of sampled fruit were quantified by high-performance liquid chromatography (HPLC). Only the drought (D; 50 mL water per application) and excess water supply (E; 260 mL water per application) treatments were applied, and all other experimental conditions were identical (except the climatic condition) to those used in 2016 and 2017. This experiment was performed using the pungent cultivars ‘Botankosho’ and 'Sapporo' primarily to analyze capsaicinoids accumulation.

Solution preparation for analysis of sugar and glutamic acid contents

Extracts for sugar and glutamic acid analyses were prepared from a known amount of fruit tissue ground using a grinder (YMB-400, Yamazen, Osaka, Japan) and filtered with 125 mm filter paper (ADVANTEC, Tokyo, Japan). Extracts were prepared for quantitative analysis of glucose (Strips- Reflctoquant® Glucose-Test, Merck KGaA, Darmstadt, Germany), total sugar (Strips- Reflctoquant® Total Sugar-Test (glucose and fructose), Merck KGaA, Darmstadt, Germany), and glutamic acid (Strips- Agrocheck Glutamic acid test, Kanto Chemical, Co., Inc., Tokyo, Japan) using a digital portable spectrophotometer (RQ flex plus 10, Merck, Darmstadt, Germany). According to Nonaka et al. (2012), glucose, total sugar, and glutamic acid contents in sweet pepper fruit detected using a RQ flex spectrophotometer and by capillary electrophoresis methods (Horie, 2009) yield approximately similar results.

Solution preparation for analysis of Brix

Brix is primarily a measure of the sugar concentration in a solution. Extracts were prepared from fruit tissue ground using a grinder (YMB-400, Yamazen) followed by filtering through 125 mm filter paper (ADVANTEC). Extracts were used directly to measure the Brix value with a digital portable refractometer (Pen-J, Atago Co. Ltd, Tokyo, Japan).

Capsaicinoids analysis - HPLC apparatus and analysis conditions

The HPLC analysis conditions were as follows: LC column (50 × 3.0 mm; Shimadzu Corporation, Kyoto, Japan), column temperature 40 °C, mobile phase 70% methanol, flow rate 1 mL/min, and absorbance at 280 nm wavelength. To examine the effectiveness of the analysis conditions, capsaicin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was used as a standard. Standard capsaicin solutions of 62.5, 125, 250, and 500 µg/mL (Othman et al., 2011) were analyzed and a calibration curve was prepared. Sample solutions was prepared by using 24 hours freeze dried chili pepper fruits, capsaicin was extracted after grinded chili pepper powder (2 mg) to the methanol (20 mL) after kept 1 hour under 40 °C.

Results

Fruit fresh weight (g)

The fresh fruit weight (g) of chili pepper fruit showed a gradual increase with increasing harvesting DAF up to 50 DAF in each treatment group of all cultivar.

In 2016, the fruit weight of ‘Manganji’ harvested at 30, 40 and 50 DAF was significantly lower in drought-treated plants than in the other groups (Fig. 2-M1). In 2017, the fruit weight of ‘Manganji’ was significantly lower in drought-treated plants, followed by the other treatments at 30 and 50 DAF (Fig. 2-M2). In ‘Manganji’ fruit harvested at 20 DAF in 2017, a significant difference was observed between the drought treated plant fruits and excess water treated plant fruits. In ‘Fushimiamanaga’ fruit, the fruit weight was significantly lower in the drought-treated plants than in the other groups at 40, and 50 DAF in 2016 and 2017 (Fig. 2-F). In 2016 and 2017, a significant difference was observed between the drought treated plant fruits and excess water treated plant fruits harvested at 20 DAF (Fig. 2-F). The fruit of ‘Botankosho’ showed similar trends, the fruit weight was significantly lower in the drought-treated plants than in the other groups at 40, and 50 DAF in 2016 (Fig. 2-B1). In 2017, a significant difference was observed between the drought treated plant fruits and excess water treated plant fruits harvested at 20 and 50 DAF (Fig. 2-B2). In ‘Sapporo’ in 2017, fresh fruit weight of chili pepper fruit gradual increase with increasing harvesting DAF (Fig. 2-S2).

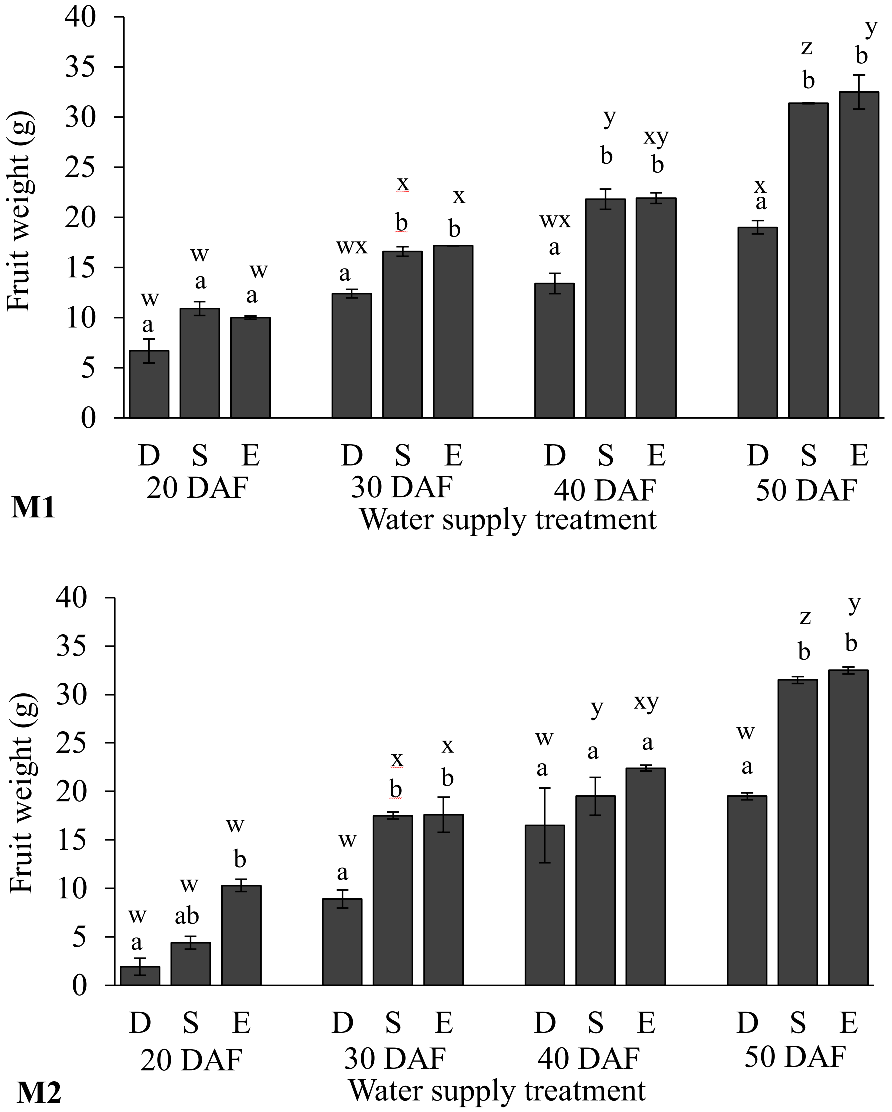


Fig. 2-M. Fruit weight (g [FW]) per fruit basis, at four stages of fruit maturation in the chili peppers 'Manganji' (M), Graphs M1 show the results of the year 2016, and M2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

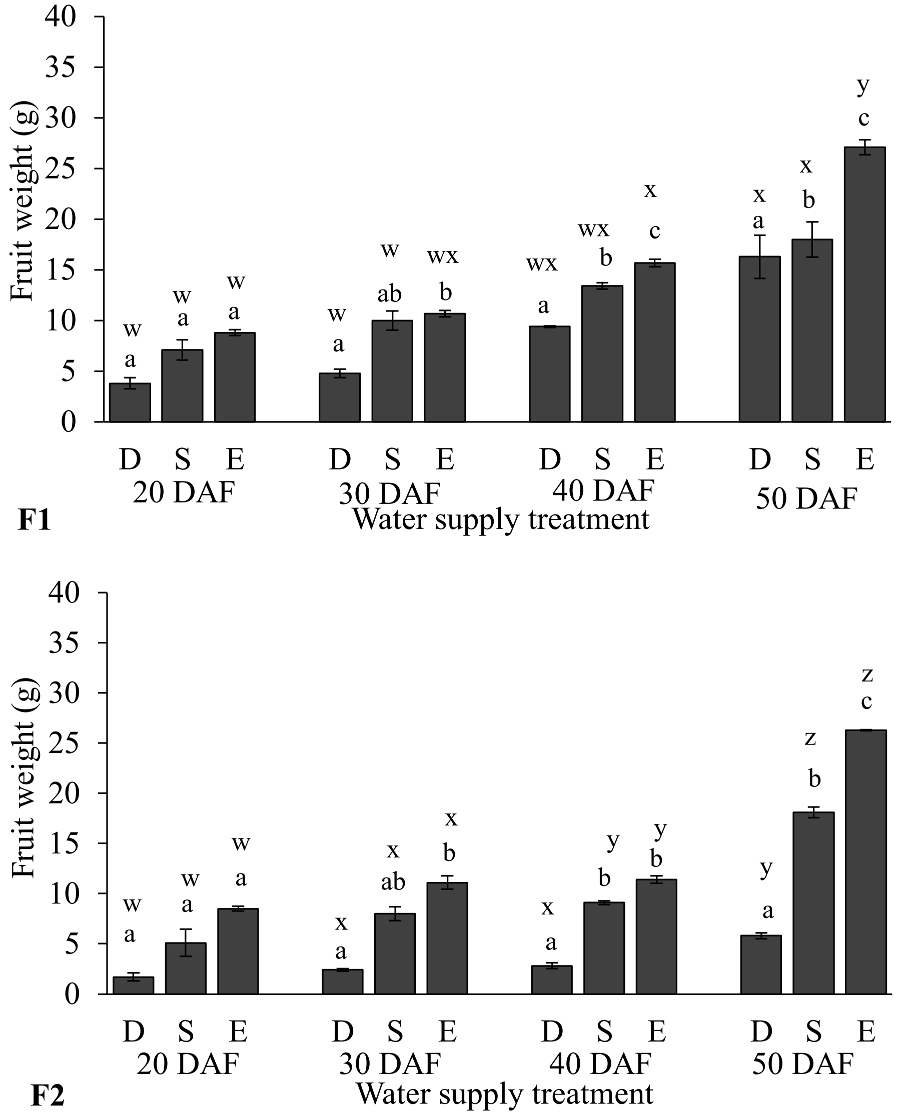


Fig. 2-F. Fruit weight (g [FW]) per fruit basis, at four stages of fruit maturation in the chili peppers ‘Fushimiamanaga’ (F), Graphs F1 show the results of the year 2016, and F2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

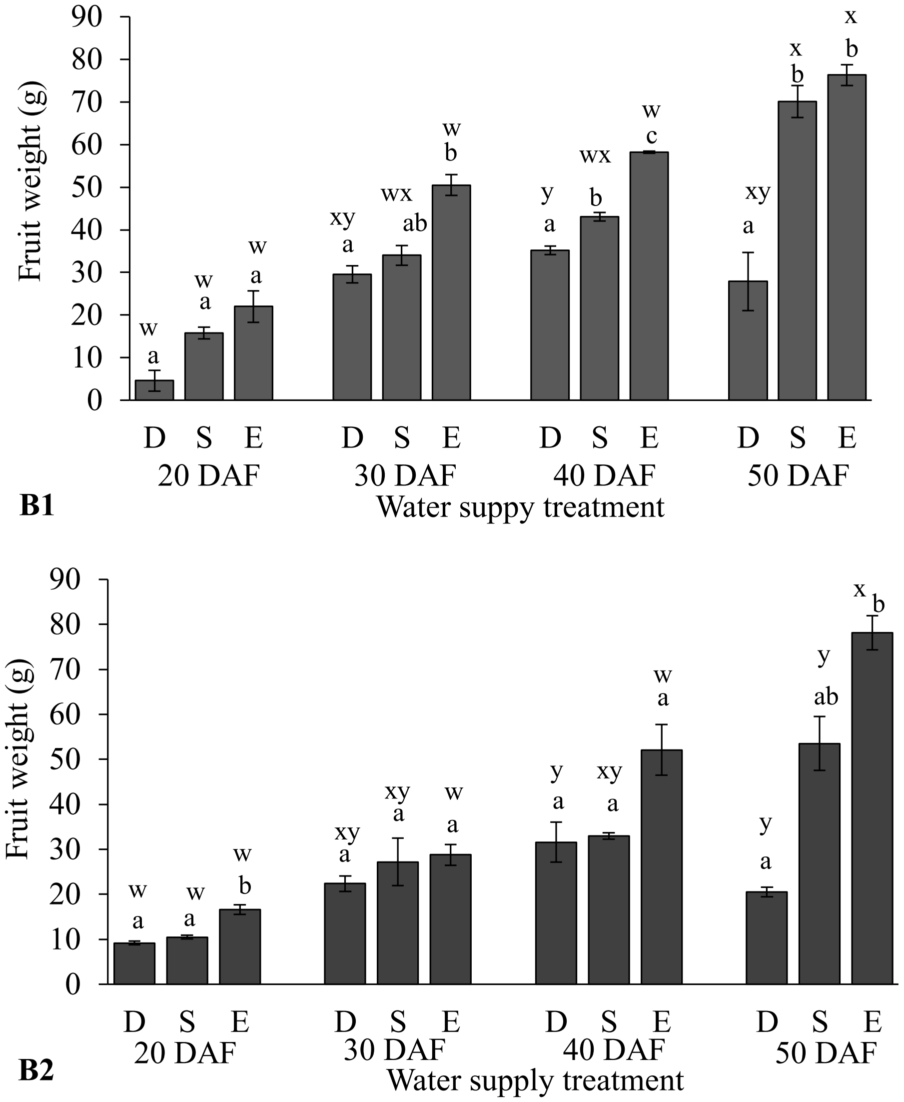
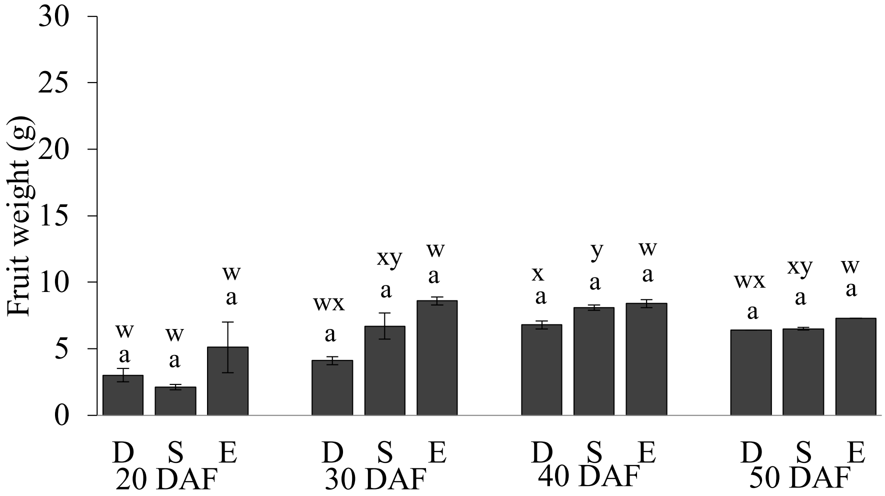


Fig. 2-B. Fruit weight (g [FW]) per fruit basis, at four stages of fruit maturation in the chili peppers 'Botankosho' (B), Graphs B1 show the results of the year 2016, and B2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.



S2

Fig. 2-S. Fruit weight (g [FW]) per fruit basis, at four stages of fruit maturation in the chili peppers ‘Sapporo’ (S), S2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

Glucose content

Glucose content, expressed on a fresh weight (FW) basis, of chili pepper fruit showed a gradual decrease up to 40 DAF in each cultivar, and there after increased slightly but not significantly at 50 DAF. In 2016, the glucose content of ‘Manganji’ differed significantly in fruit harvested at 30 and 40 DAF from drought-treated plants, and also significantly differed between the drought and excess water supply treatments at 20 and 50 DAF (Fig. 3-M1). In 2017, the glucose content of ‘Manganji’ showed a significant difference at 40 and 50 DAF in each of the three water supply treatments; in addition, fruit from the drought treatment showed a lower glucose content and fruit from the excess water supply treatment showed a higher glucose content (Fig. 3-M2). In ‘Manganji’ fruit at 20 DAF, a significant difference between the drought and excess water supply treatments was observed in 2017. In ‘Fushimiamanaga’ fruit, glucose contents were significantly lower in the drought treatment from the excess water supply treatment at 40 and 50 DAF in 2016 (Fig. 3-F1) and fruit harvested from the other treatments at 20 and 30 DAF in 2017 (Fig. 3-F2). In 2017, ‘Fushimiamanaga’ fruit harvested at 50 DAF, a significant difference in glucose content between the drought and excess water supply treatments was observed. Fruit of ‘Botankosho’ showed similar trends, with significant differences in glucose content observed in drought-treated fruit harvested at 30, 40, and 50 DAF in 2016 (Fig. 3-B1) and at 40 and 50 DAF in 2017 (Fig. 3-B2). Also in 2017, ‘Botankosho’ fruit harvested at 30 DAF, a significant difference in glucose content between the drought and excess water supply treatments was observed. Fruit of ‘Sapporo’ showed a significantly lower glucose content under drought stress at 30, 40, and 50 DAF (Fig. 3-S2).

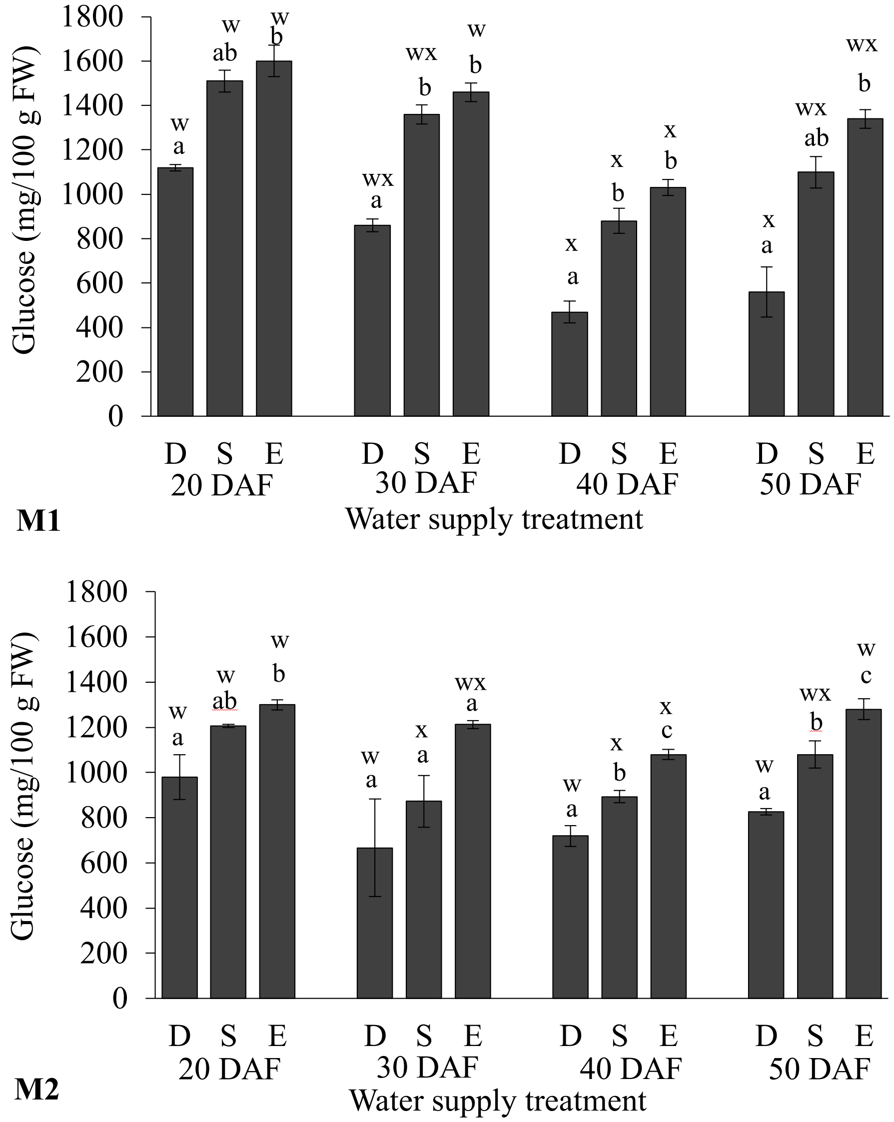


Fig. 3-M. Glucose content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili peppers 'Manganji' (M), Graphs M1 show the results of the year 2016, and M2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w and x, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

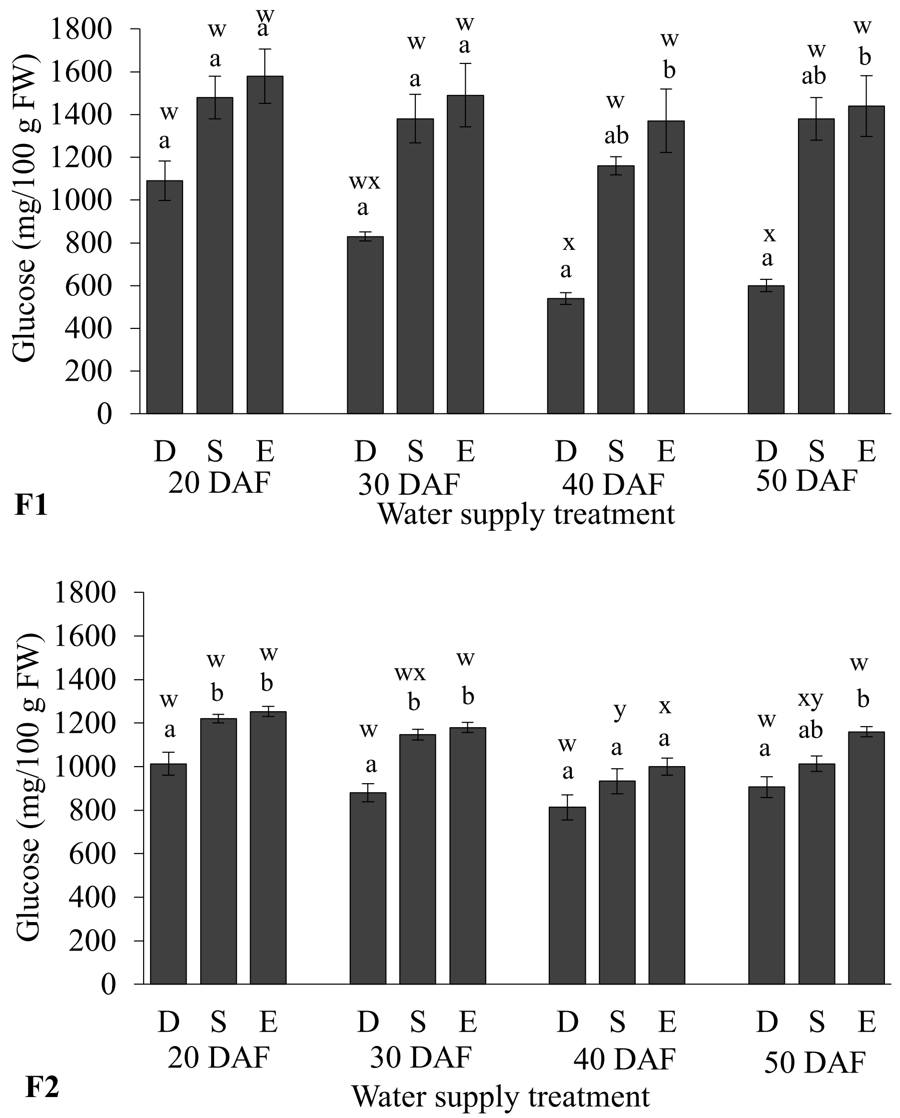


Fig. 3-F. Glucose content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili peppers ‘Fushimiamanaga’ (F), Graphs F1 show the results of the year 2016, and F2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, *P* < 0.05). Error bars indicate the standard error.

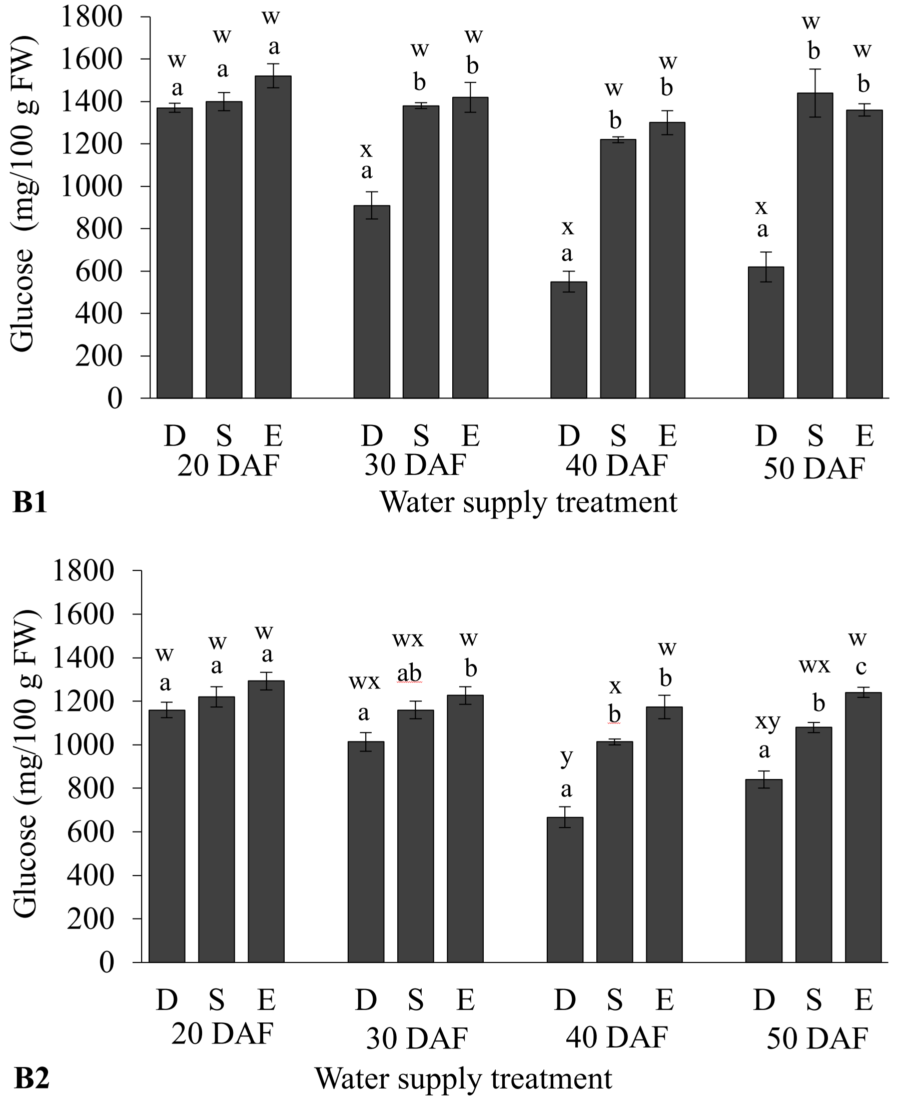


Fig. 3-B. Glucose content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili peppers ‘Bothankosho’ (F), Graphs B1 show the results of the year 2016, and B2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, *P* < 0.05). Error bars indicate the standard error.

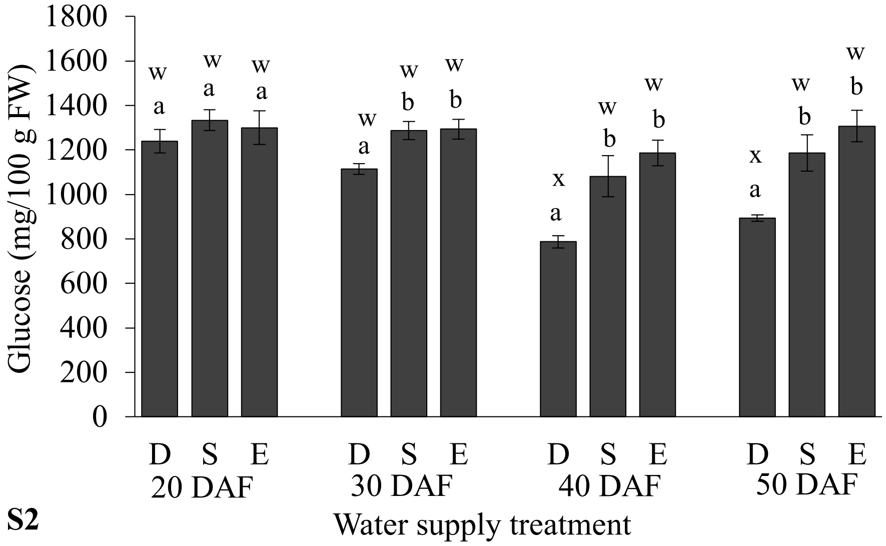


Fig. 3-S. Glucose content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili peppers ‘Sapporo’ (S), Graph S2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, and x, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, *P* < 0.05). Error bars indicate the standard error.

Total sugar content

Total sugar content, expressed on a FW basis, increased with progression of fruit maturation in all cultivars. The highest total sugar content was observed in the drought treatment in fruit harvested at 50 DAF. The content of total sugar was increased in the drought treatment compared with that observed in the excess water supply treatment. Interestingly, this pattern showed the opposite trend to glucose content in the same cultivar.

Fruit of ‘Manganji’ showed significantly higher total sugar content in drought-treated plants than in the excess water supply treatment at 40 and 50 DAF in 2016 (Fig. 4-M1) and at all DAF in 2017 (Fig. 4-M2). The total sugar content behavior of ‘Fushimiamanaga’ fruit showed identical trends with the stress condition. The total sugar content of ‘Fushimiamanaga’ differed significantly between the drought and excess water supply treatment at 30 and 50 DAF in 2016 (Fig. 4-F1) and at each DAF in 2017 (Fig. 4-F2). In 2016, fruit of ‘Botankosho’ harvested at 20, 40 and 50 DAF showed significant differences in total sugar contents between the drought and excess water supply treatments (Fig. 4-B1). ‘Botankosho’ fruit showed no significant differences in total sugar content among the water supply treatments except at 40 DAF in 2017 (Fig. 4-B2). The fruits of ‘Sapporo’ harvested at 30, 40, and 50 DAF in the drought treatment showed significantly higher total sugar content than that of the other treatments (Fig. 4-S2).

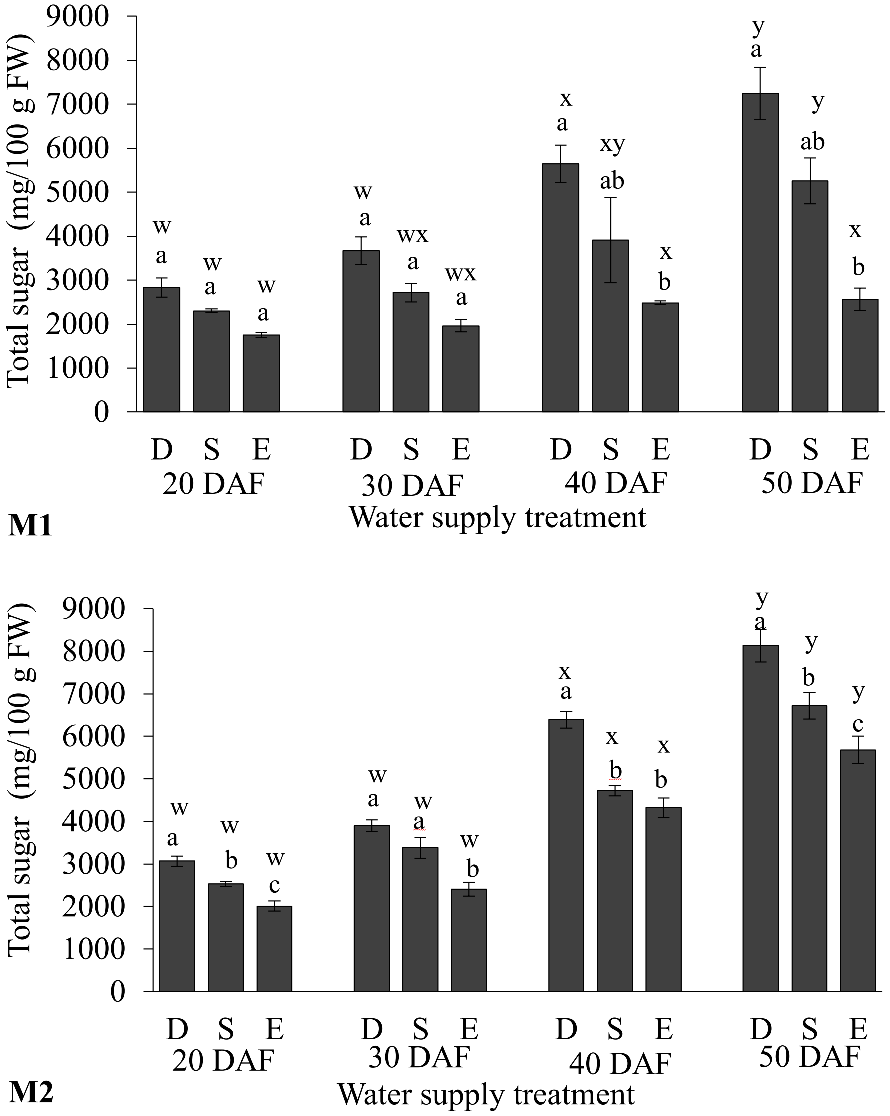


Fig. 4-M. Total sugar content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili peppers ‘Manganji’ (M), Graphs M1 show the results of the year 2016, and M2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

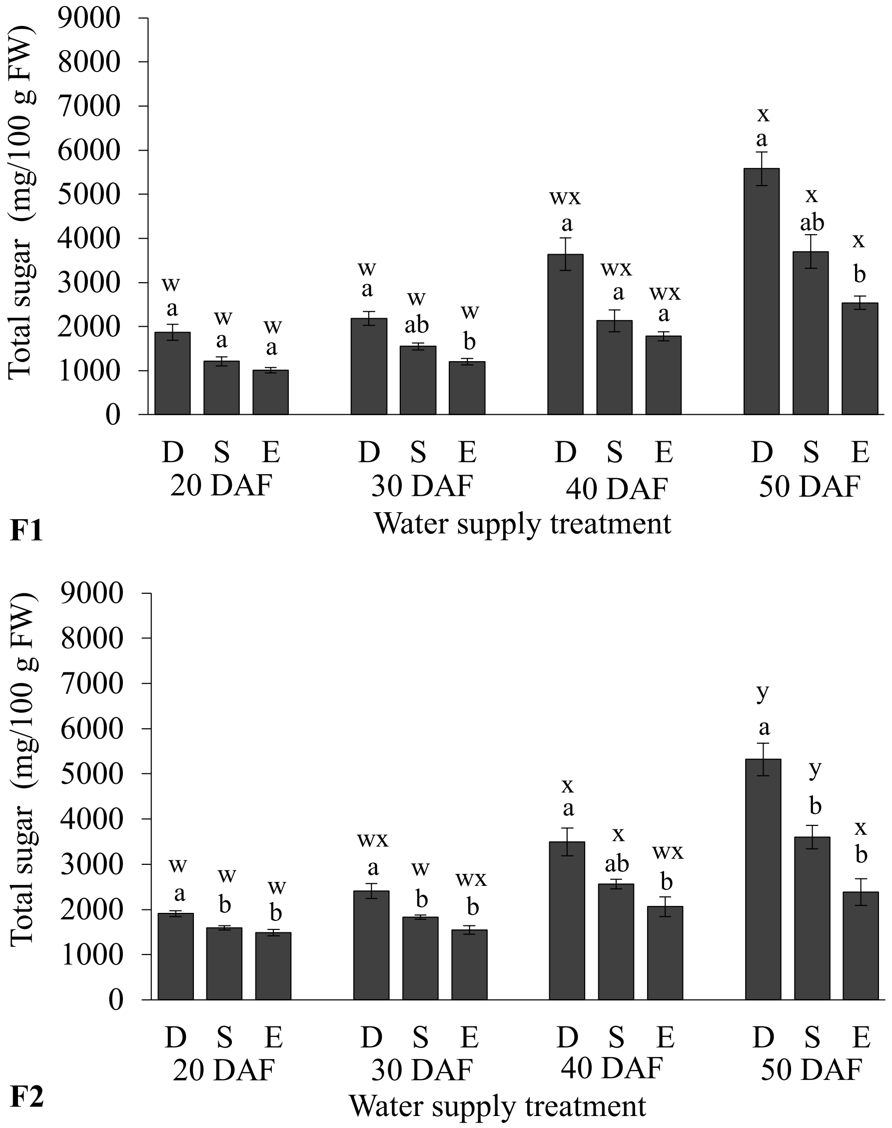


Fig. 4-F. Total sugar content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili peppers ‘Fushimiamanaga’ (F), Graphs F1 show the results of the year 2016, and F2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

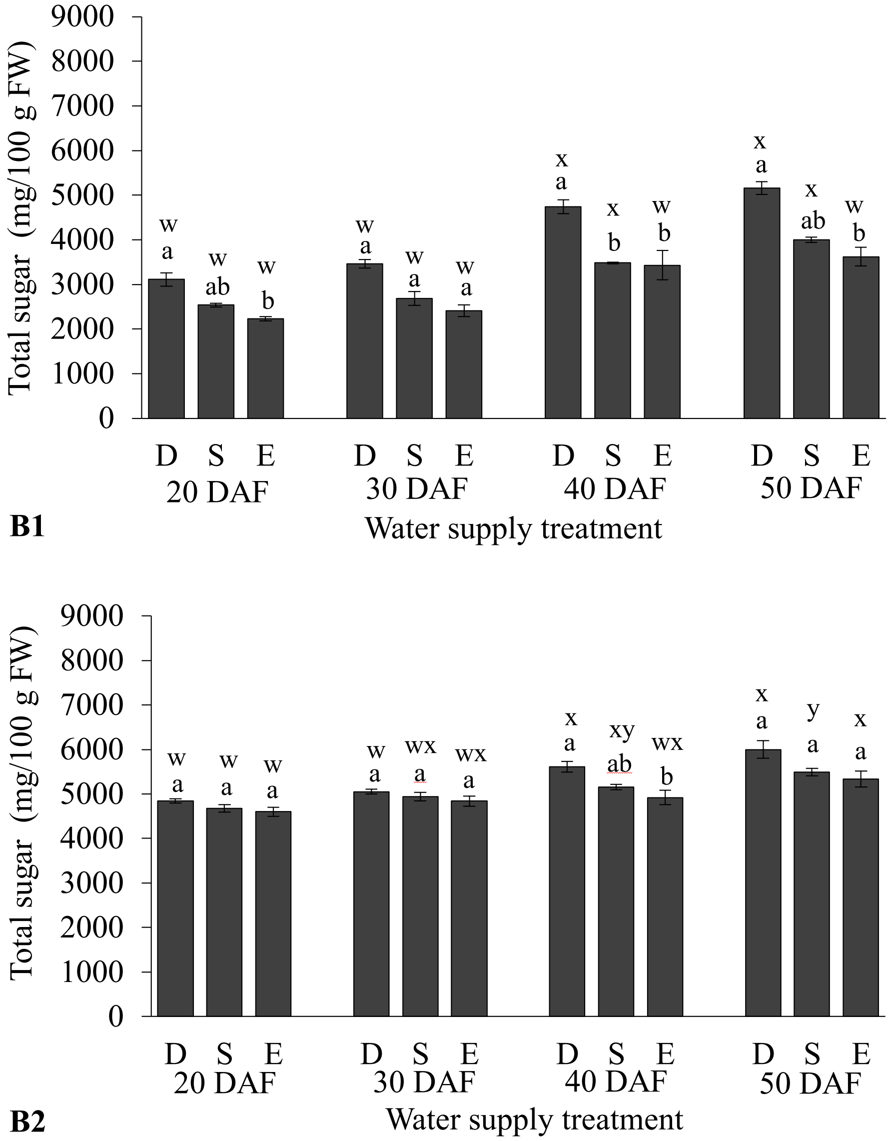
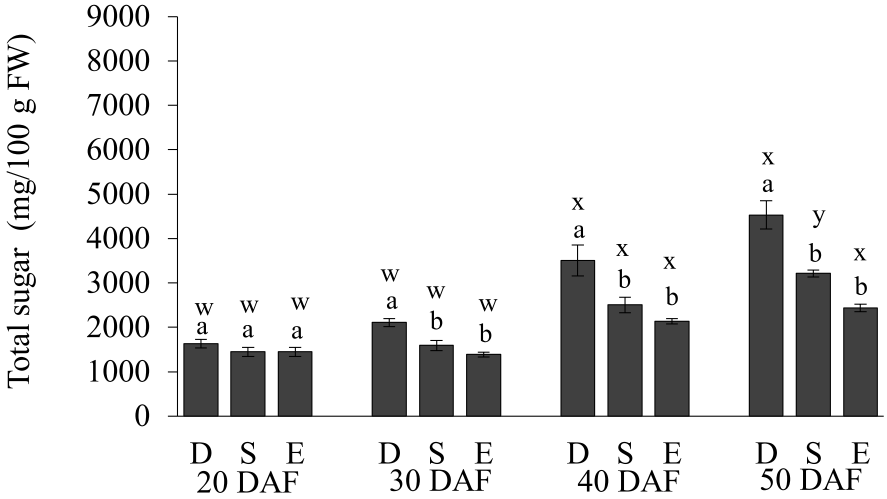


Fig. 4-B. Total sugar content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili peppers ‘Botankosho’ (F), Graphs B1 show the results of the year 2016, and B2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.



S2

Fig. 4-S. Total sugar content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili peppers 'Sapporo' (F), Graphs S2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

Brix

The fruit Brix percentage of all four cultivars tend to increase with reduction in the amount of water applied and with delay in harvesting. The highest Brix percentage was observed in the drought treatment at an advanced stage of maturation (50 DAF).

Significant differences in Brix under excess water supply and drought stress was observed in ‘Manganji’ fruit at 20 DAF in 2016 (Fig. 5-M1) and at 30, 40, and 50 DAF in 2017 (Fig. 5-M2). Variation in Brix was similar in the other three cultivars. In ‘Fushimiamanaga’, fruit in the drought treatment at 30 and 40 DAF differed significantly in Brix from that of the other treatments in 2016 (Fig. 5-F1). In 2017, fruit harvested at 40 DAF from plants treated with excess water supply differed significantly in Brix from that of the other treatments. In the same year (2017), fruit harvested at 50 DAF in the drought treatment showed significantly higher Brix compared with that of the other treatments (Fig. 5-F2). At 50 DAF in 2016 and 20 DAF in 2017, a significant difference in Brix was observed between the drought and excess water supply treatments in ‘Fushimiamanaga’ fruits. In ‘Botankosho’, fruit harvested at 20, 30, and 40 DAF in 2016 (Fig. 5-B1) and 20 and 30 DAF in 2017 showed significantly higher Brix in the drought treatment. Fruit harvested at 40 DAF from plants cultivated under excess water supply showed significantly lower Brix in 2017. A significant difference was observed in the Brix of fruit from the drought and excess water supply treatments at 50 DAF in 2017 (Fig. 5-B2). Fruit of ‘Sapporo’ showed similar variation in Brix to that of the other cultivars and significantly lower Brix was observed in the excess water treatment at 40 DAF. In addition, significant difference in the Brix of fruit from the drought and excess water supply treatments was observed at 30 and 50 DAF in 2017 (Fig. 5-S2).

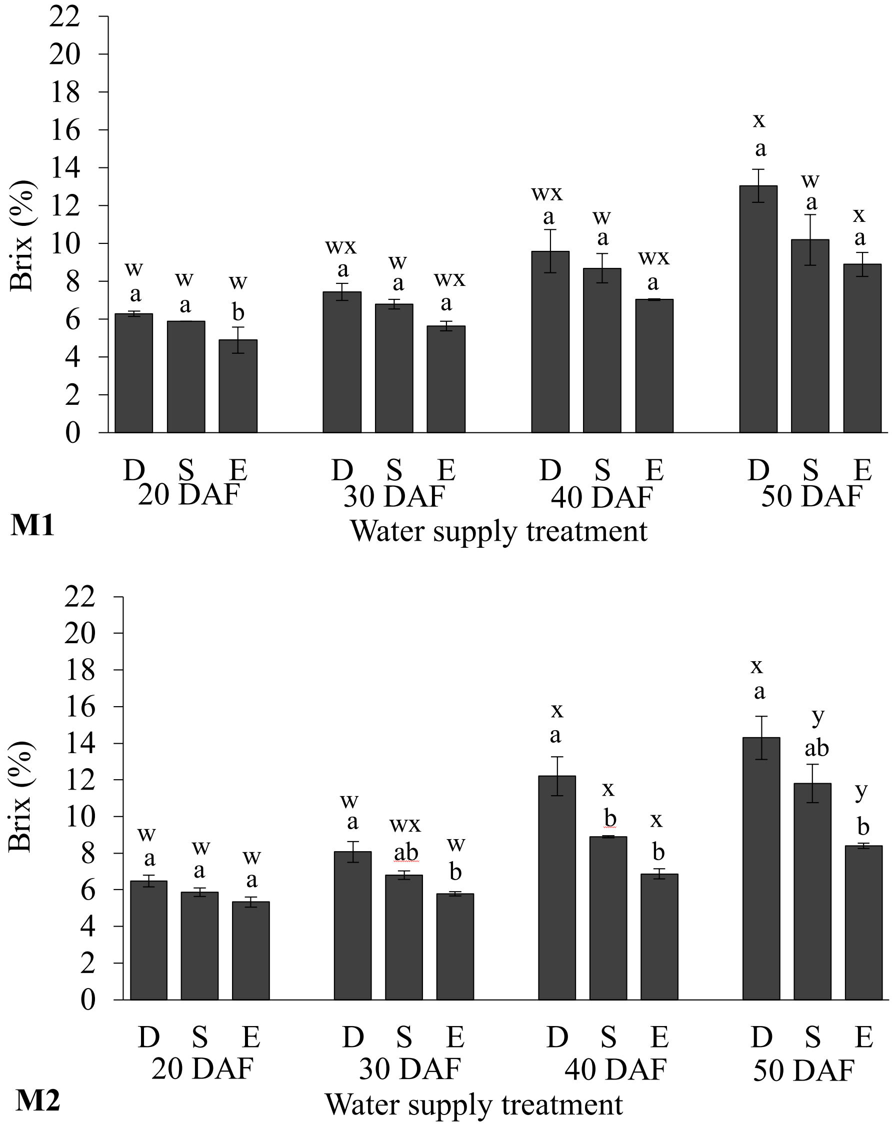


Fig. 5-M. Brix at four stages of fruit maturation in the chili peppers 'Manganji' (M), Graphs M1 show the results of the year 2016, and M2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

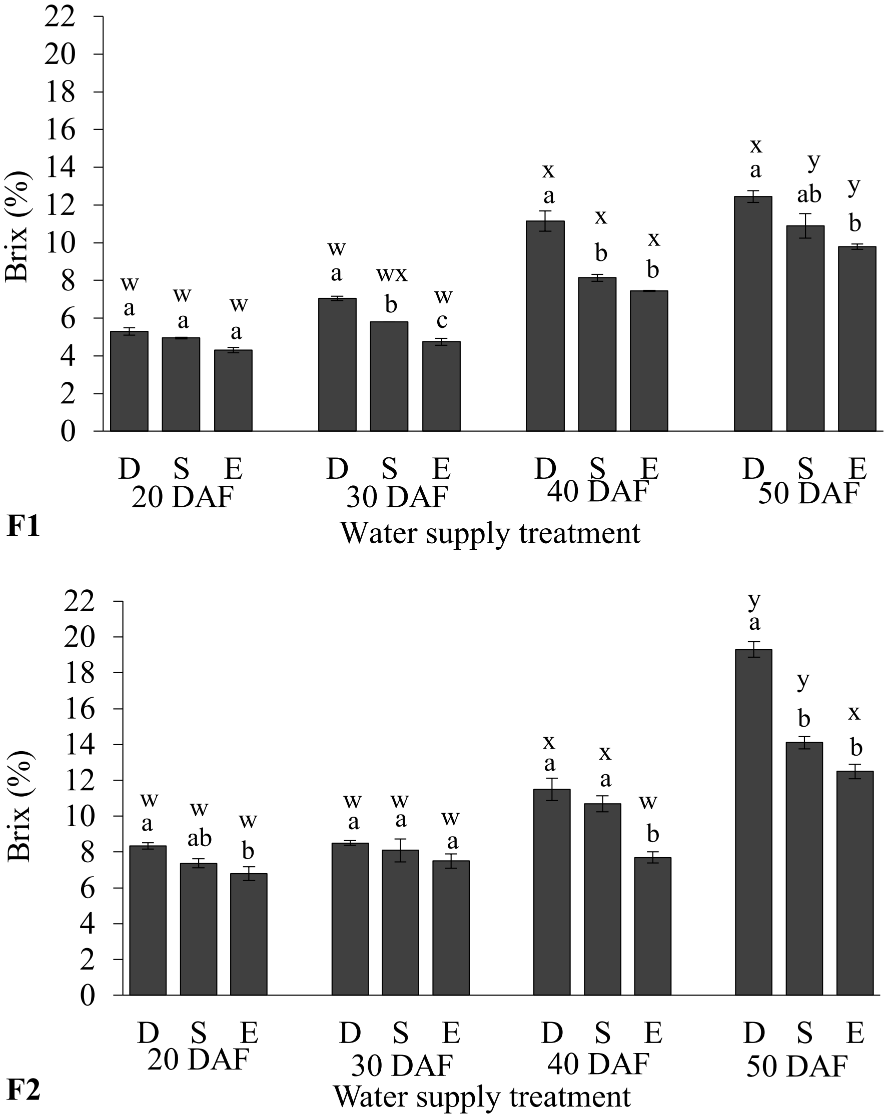


Fig. 5-F. Brix at four stages of fruit maturation in the chili peppers 'Fushimiamanaga' (F), Graphs F1 show the results of the year 2016, and F2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, *P* < 0.05). Error bars indicate the standard error.

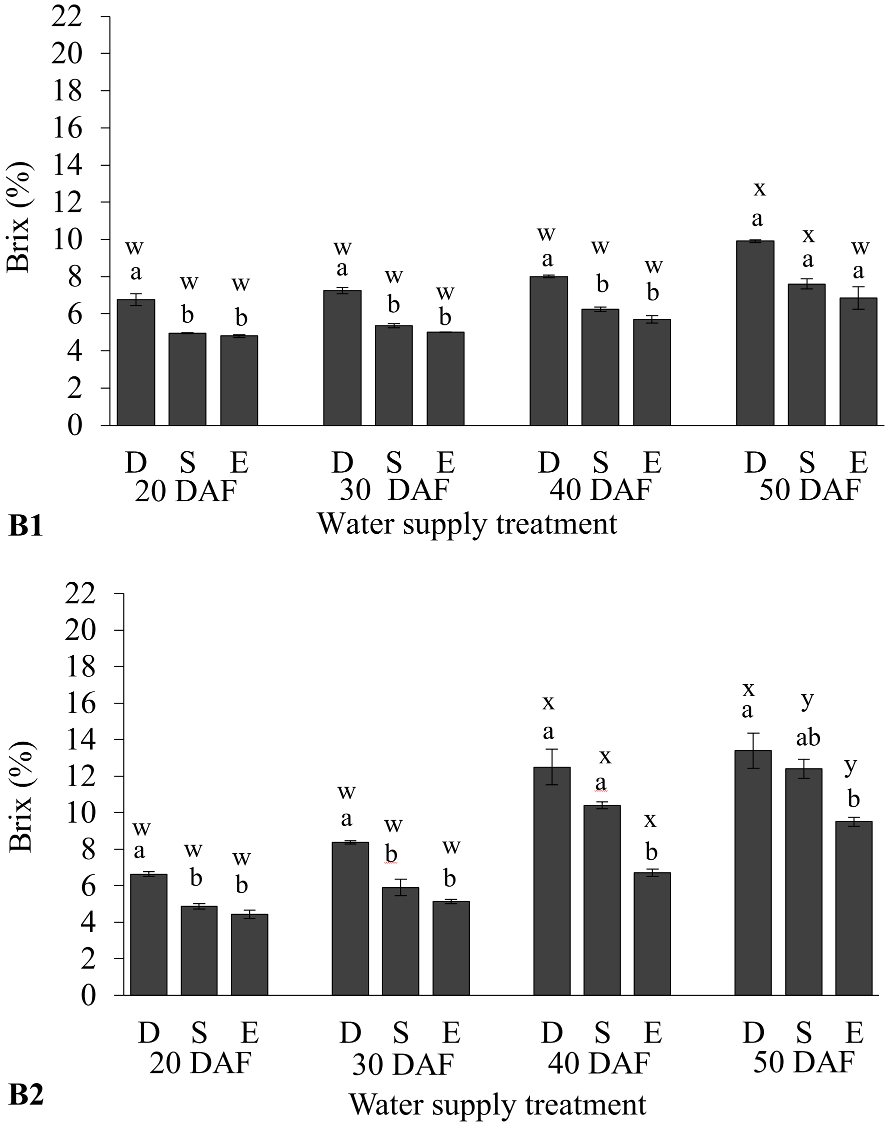
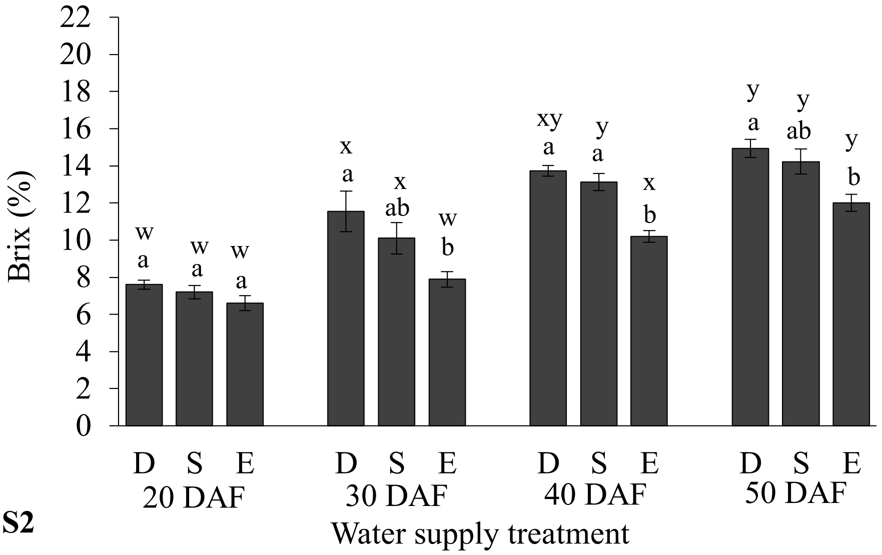


Fig. 5-B. Brix at four stages of fruit maturation in the chili peppers 'Botankosho' (B), Graphs B1 show the results of the year 2016, and B2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

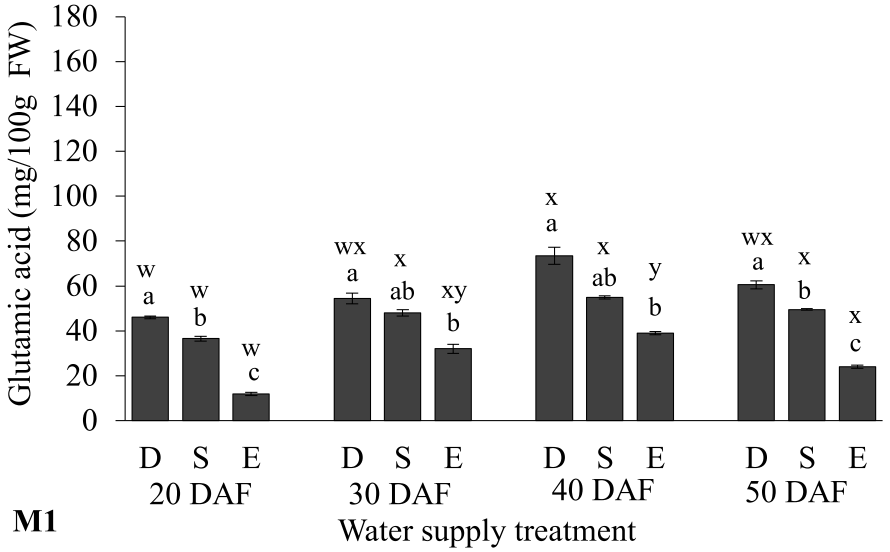


S2

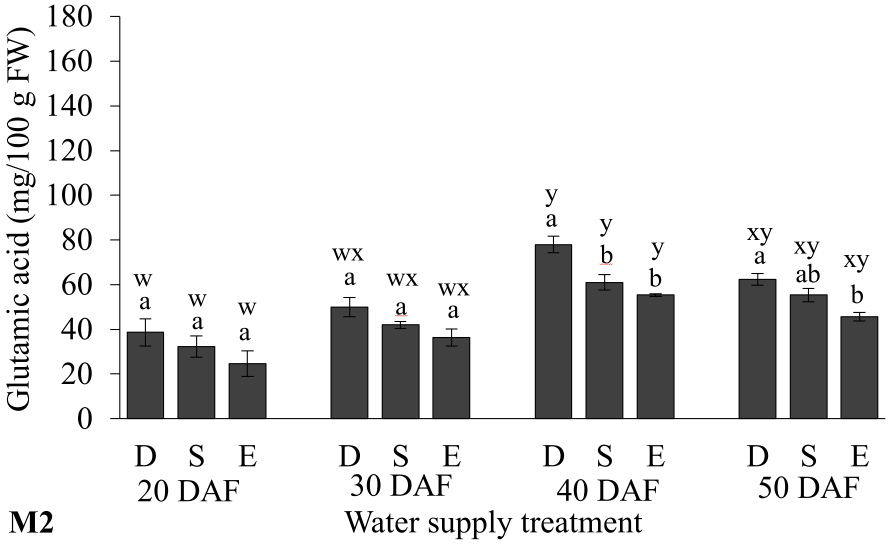
Fig. 5-S. Brix at four stages of fruit maturation in the chili peppers 'Sapporo' (S), Graphs S2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

Glutamic acid content

Glutamic acid content, expressed on a FW basis, increased in each cultivar with delay in harvesting up to 40 DAF. However, in the majority of cases the glutamic acid content showed no significant difference between fruit harvested at 40 and 50 DAF, except for 'Fushimiamanaga' fruit in 2017 (Fig. 6-F2). In addition, all cultivars showed higher contents of glutamic acid in the drought treatment compared with that of the other water supply treatments. In 'Manganji' a significant difference in glutamic acid content was observed between the drought and excess water supply treatments at all DAF in 2016 (Fig. 6-M1) and at 40 and 50 DAF in 2017 (Fig. 6-M2). In 2016, significantly lower glutamic acid content was observed in the excess water treatment compared with that of the drought treatment in 'Fushimiamanaga' except for fruit harvested at 20 DAF. Interestingly, in 'Fushimiamanaga', glutamic acid content was significantly higher in fruit of drought-treated plants at 30, 40, and 50 DAF compared with the other water supply treatments in 2017 (Fig. 6-F2). And in same year, significantly lower glutamic acid content was observed in fruits from the excess water treated plant than other water supply treatments in 20 DAF. In 'Botankosho' fruit, glutamic acid content was significantly higher in the drought treatment at 20, 40, and 50 DAF in 2016 (Fig. 6-B1), and at 20, 30, and 40 DAF in 2017 (Fig. 6-B2), compared with that of the other water supply treatments. Significant difference in glutamic acid content between the drought and excess water supply treatments was observed in 'Botankosho' fruit at 50 DAF in 2017. The glutamic acid content in 'Sapporo' fruit was significantly lower in the excess water supply treatment at 40 DAF. In addition, significant differences between the drought and excess water supply treatments was observed in 'Sapporo' fruit at 30 and 50 DAF (Fig. 6-S2).



M1



M2

Fig. 6-M. Glutamic acid (mg/100 g FW) at four stages of fruit maturation in the chili peppers 'Manganji' (M), Graphs M1 show the results of the year 2016, and M2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

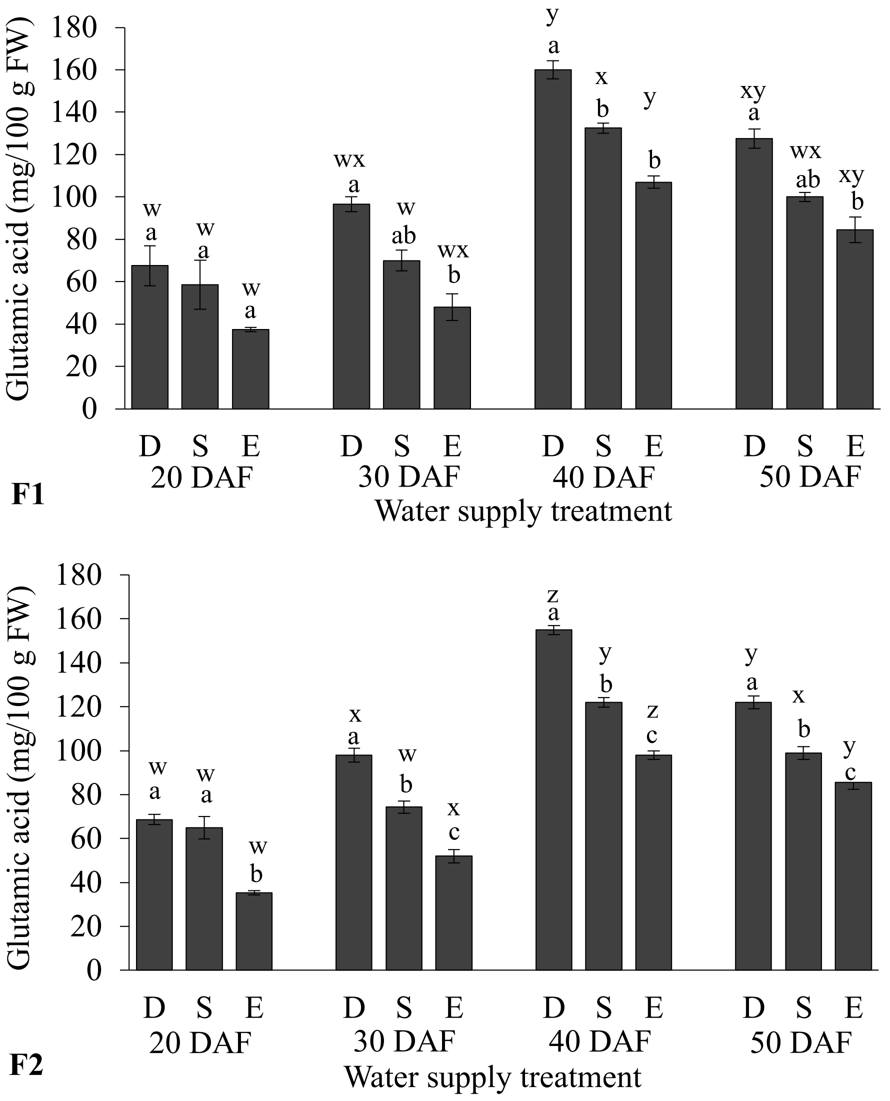


Fig. 6-F. Glutamic acid (mg/100 g FW) at four stages of fruit maturation in the chili peppers ‘Manganji’ (M), Graphs M1 show the results of the year 2016, and M2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

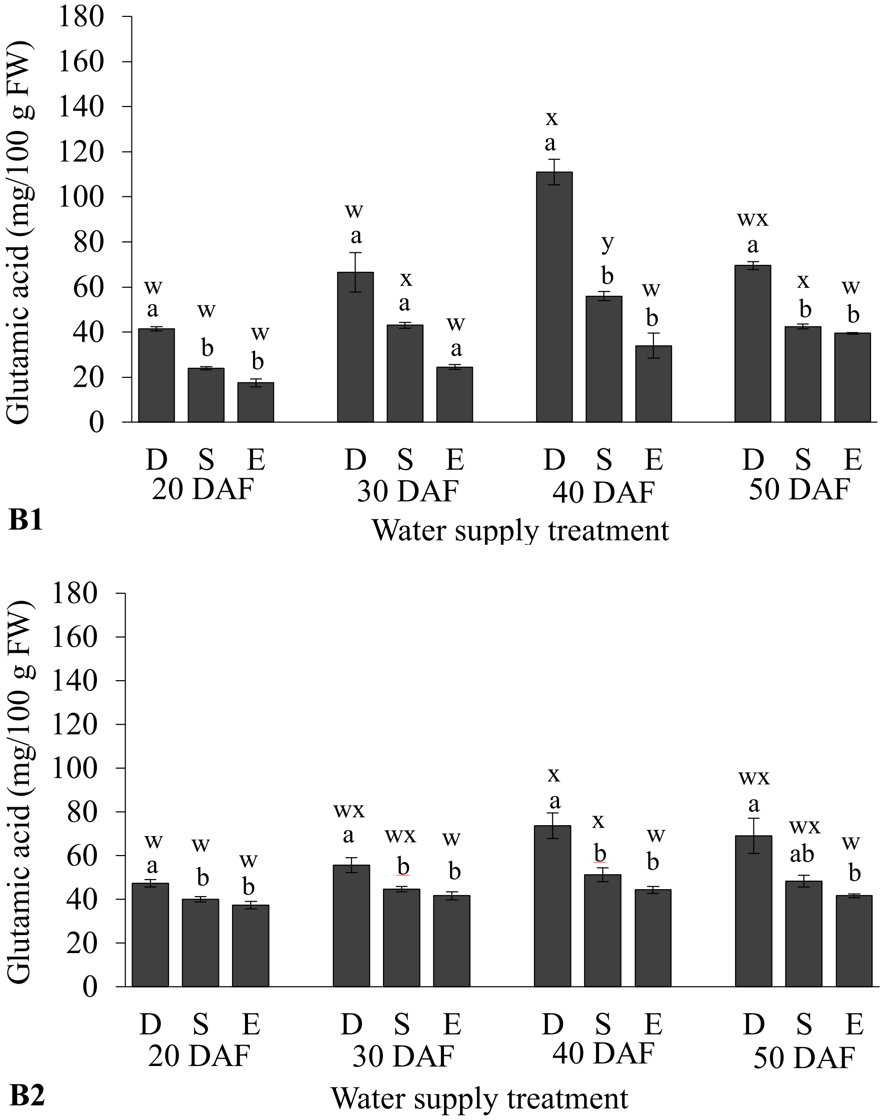


Fig. 6-B. Glutamic acid (mg/100 g FW) at four stages of fruit maturation in the chili peppers ‘Manganji’ (M), Graphs M1 show the results of the year 2016, and M2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, *P* < 0.05). Error bars indicate the standard error.

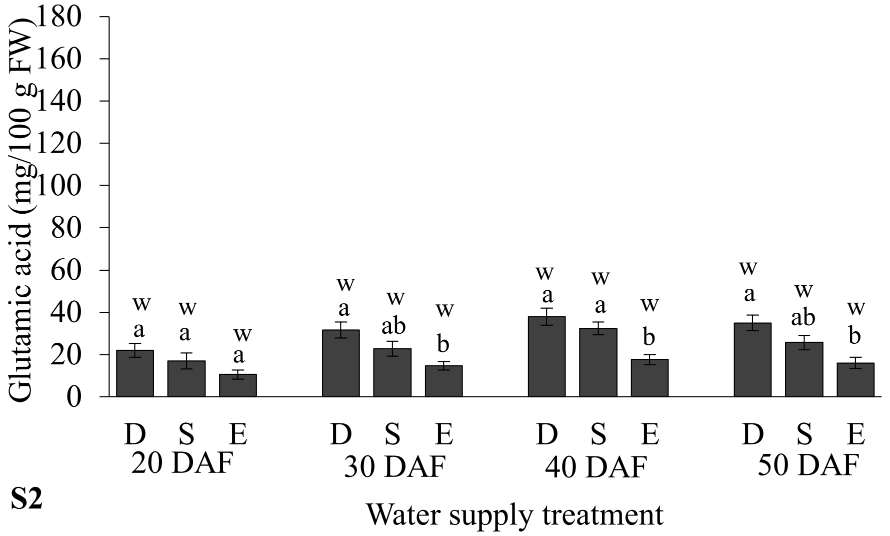


Fig. 6-S. Glutamic acid (mg/100 g FW) at four stages of fruit maturation in the chili peppers ‘Manganji’ (M), Graphs M1 show the results of the year 2016, and M2 show the results of the year 2017. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and lowercase letter w, for the same treatments (drought, D; standard water supply, S; and excess water supply, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

Capsaicinoid content

Capsaicinoids were detected only in ‘Botankosho’ and ‘Sapporo’ fruit. Patterns of capsaicinoid content tended to be similar to those of total sugar content. Capsaicinoid content increased with delay in harvesting of the fruit and with reduction in water supply from excess to drought. ‘Botankosho’ fruit showed significantly higher capsaicinoid content in the drought treatment at 40 and 50 DAF in 2016 (Fig. 7-B1). In 2017, capsaicinoid content in the drought treatment at 20, 40 and 50 DAF differed significantly from other treatments (Fig. 7-B2). In addition, a significant difference was observed between the drought and excess water supply treatments at 30 DAF in 2017. A significant difference in capsaicinoid content of ‘Sapporo’ fruit was observed in the drought treatment at all DAF compared with that of the other treatments in 2017 (Fig. 7-S2).

The capsaicinoid contents of the placenta of ‘Botankosho’ and ‘Sapporo’ fruit, recorded in the 2018 experiment, were consistent with the patterns observed for the whole fruit. A significant difference in capsaicinoid content was observed in the drought treatment compared with that of the excess water supply treatment at all DAF (Fig. 8-B, 8-S).

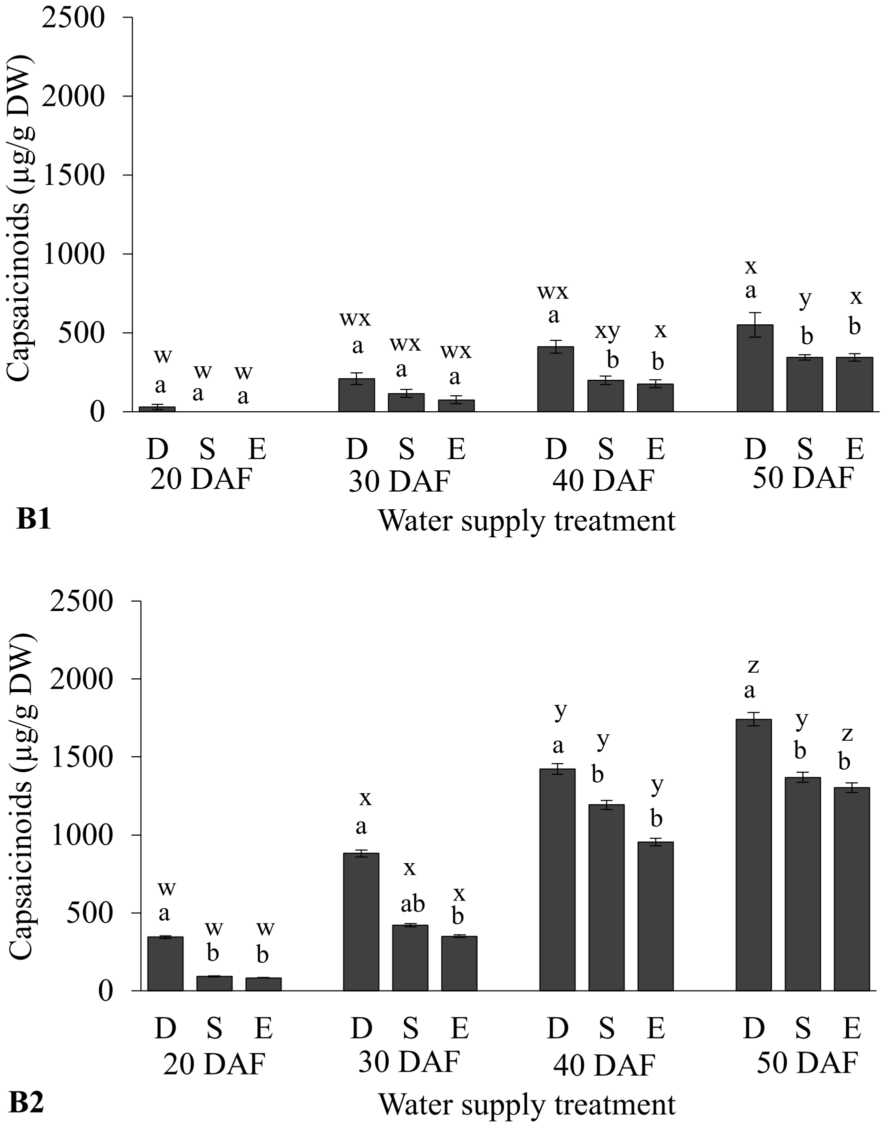
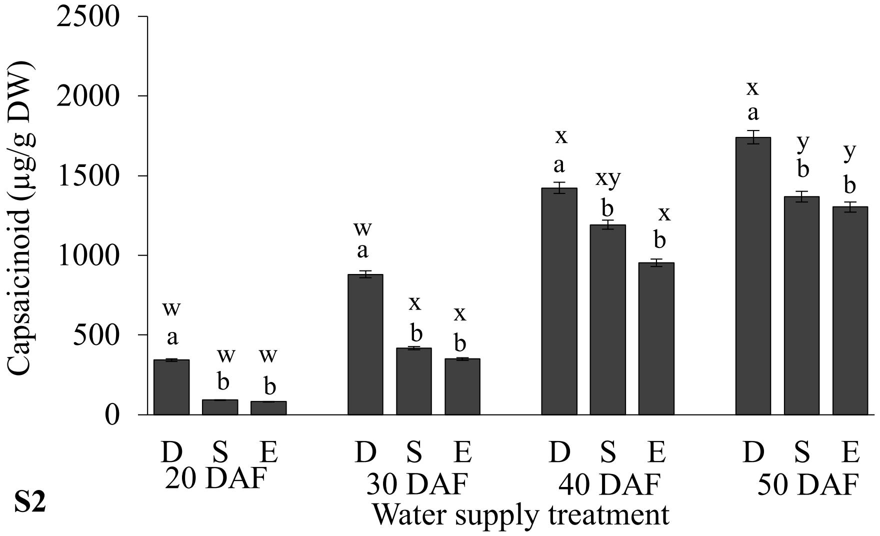


Fig. 7-B. Capsaicinoid content (µg/g dry weight [DW]) at four stages of fruit maturation in chili pepper 'Botankosho' (B). Graph B1 represent the year 2016 and B2 represent year 2017. Fruit were sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lower-case letters a and b, for the same DAF, and different lower-case letters w, x, y, and z, for the same treatment (drought, D; standard water supply, S; and excess water supply, E), indicate significant differences between treatments and between DAF, respectively (Tukey's pairwise test, *P* < 0.05). Error bars indicate the standard error.



S2

Fig. 7-S. Capsaicinoid content (µg/g dry weight [DW]) at four stages of fruit maturation in chili pepper 'Sapporo Oonaga Nanban' (S). Graph S2 represent year 2017. Fruit were sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lower-case letters a and b, for the same DAF, and different lower-case letters w, x, and y, for the same treatment (drought, D; standard water supply, S; and excess water supply, E), indicate significant differences between treatments and between DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

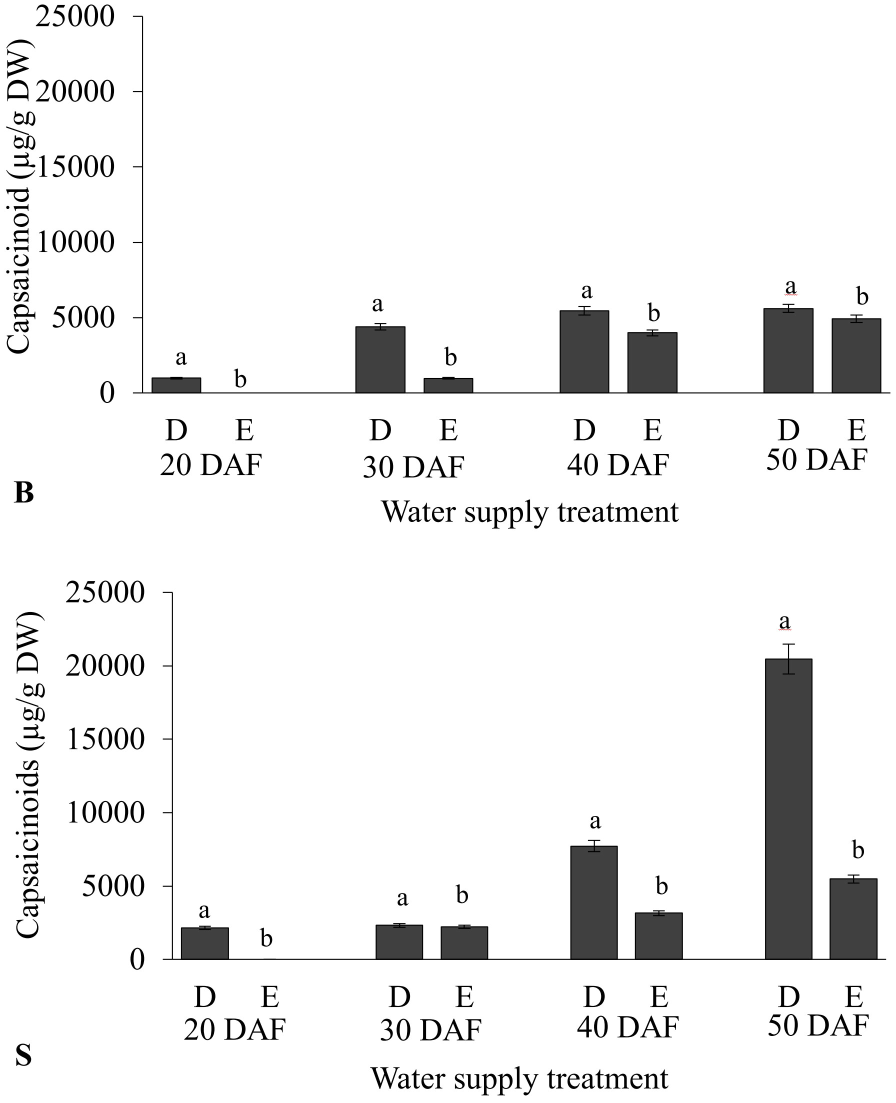


Fig. 8. Capsaicinoid content of the placenta (µg/g dry weight [DW]) at four stages of fruit maturation in chili pepper 'Botankosho' (B) and 'Sapporo Oonaga Nanban' (S) in 2018. Fruit were sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lower-case letters a and b, for the same DAF indicate significant differences between treatments (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

Discussion

The size of chili pepper fruits increased with development and growth ceased at < 30 DAF. The color of the pericarp changed from green to red coincident with fruit maturation at around 40 DAF. But, the color of the pericarp of ‘Sapporo’ changed at < 40 DAF. This sequence was common to all cultivars and treatments in the present study (Fig. 9). According to Minami et al. (1998), the fruits of *Capsicum annuum* ‘Takanotsume’ plants, grown in the same experimental field of Shinshu University as that used in the current research, stopped growing by < 20 DAF and turned red from 50 to 60 DAF. Comparing the present results with those of this previous study, fruit enlargement was slower and the change in pericarp color to red was earlier in the present investigation. The difference in timing of cessation of fruit growth is considered to be because ‘Takanotsume’ fruit are smaller than those of the cultivars used in the present study. The difference in the timing of change in fruit color is likely because the plants were grown in a greenhouse in the present study and in cool, outdoor fields in the study by Minami et al. (1998).

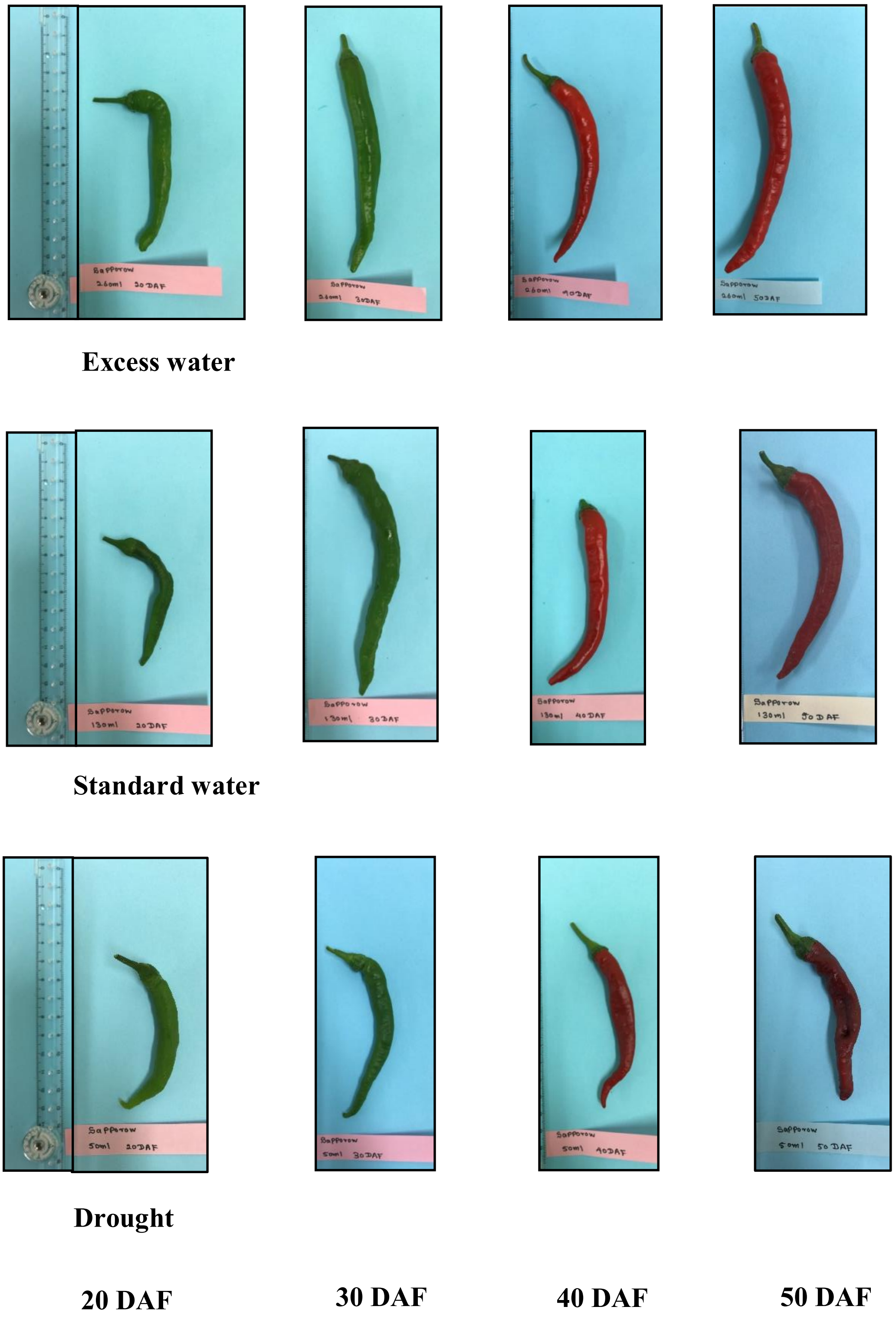


Fig. 9. Fruit size at four stages of maturation in chili pepper ‘Sapporo Oonaga Nanban’ grown under three water supply treatments. Fruit were sampled at 20, 30, 40, and 50 days after flowering (DAF).

Total sugar content and Brix of the fruit tended to increase with delay in harvesting, but the glucose content showed only limited changes with increase in DAF. Given that the highest total sugar content recorded among all treatments, cultivars, and DAF sampling points was about 8000 mg, compared with the highest glucose content of about 1600 mg, the present results showed that the ratio of glucose content to total sugar content was small. In addition, the sucrose content is extremely low in fruit of sweet and pungent peppers (Ministry of Education, Culture, Sports, Science and Technology, https://fooddb.mext.go.jp/index.pl/, March 15, 2020), therefore it is predicted that the ratio of sucrose content to total sugar content is also extremely low. These results indicated that the majority of the sugars contained in chili pepper fruits were fructose, and the ratio of fructose content to total sugar content is expected to increase as the fruit matures. In the case of cherry tomato, the total sugar content increases with fruit maturation, but the glucose content also increases (Hayata et al., 1998). Consequently, I surmise that sugar metabolism and accumulation differ in tomato fruit and chili pepper fruit. The glutamic acid content in the fruit increased up to 40 DAF, and thereafter remained unchanged or decreased. According to Nonaka et al. (2012), the glutamic acid content of red fruit (< 60 DAF) of 'Botankosho' is three times higher than that of green fruit (< 50 DAF). The inconsistency of the present results with those of Nonaka et al. (2012) may be because the latter authors analyzed fruit from 'Botankosho' plants grown in open fields at high altitude. With regard to fruit color, the present results are consistent with those of Nonaka et al. (2012), because red fruit (equivalent to 40 DAF in the present study) showed significantly higher glutamic acid content than that of green fruit (equivalent to 30 DAF in the present study). However, in the current study the glutamic acid content of red fruit was not more than three times higher than that of green fruit.

The capsaicinoid content increased with fruit development. A previous report showed that capsaicinoid content increases until just before the fruit turns red and then decreases after the pericarp has turned red (Minami et al., 1998). In the present study, capsaicinoid content did not decrease even after 40 DAF, when the fruit had turned red, and no significant difference was observed among the majority of treatments from 50 DAF. It is possible that the discordance in results between studies was due to the difference in cultivars used.

Changes in the glucose content and total sugar content with fruit growth showed opposite trends. Similarly, with consideration of the amount of water supplied, the changes in glucose content and total sugar content showed conflicting responses. Increase in water supply caused an increase in glucose content and decrease in total sugar content. These results imply that by increasing water availability, the fructose content was reduced, but the ratio of glucose content to total sugar content was small. In cherry tomato, increase in water supply decreases the total sugar content as well as glucose content (Hayata et al., 1998). Thus, it is speculated that sugar metabolism and accumulation differ in tomato fruit and chili pepper fruit.

In tomato fruit, total sugar content and Brix increase in response to drought (Nahar and Gretzmacher, 2002). The soluble solid content (Brix) in tomato shows a strongly adverse relationship with yield under drought. Plants with low water supply are able to support only about 20% of the potential yield, thus the highest Brix is observed under low water supply. This was considered to be due to the fact that if the number of developing fruit is low, the amount of sugars photosynthesized in the leaves that are distributed to individual fruit increases (Bakr et al., 2016). However the results of the present study supported these previous reports on tomato because there was a tendency for reduction in the number of leaves and fruit on plants in the drought treatment compared with those in the other water supply treatments, I did not record yield in this experiment due to harvest many immature fruit in the present research to focus on taste component behavior when fruit growth. Therefore, it is necessary to investigate the relationship between yield, leaf number, photosynthetic ability, and sugar content in more detail in the future.

The relationship between glutamic acid content in the fruit and water supply was consistent with the relationships observed for total sugar content and Brix. According to the Umami Information Center (https://www.umamiinfo.com/richfood/foodstuff/tomato.html/, March 11, 2020), tomato fruit contain high contents of the umami provider glutamic acid, especially as the fruit ripens. However, chili pepper tended to have the highest glutamic acid content at 40 DAF in the present study.

Capsaicinoid content also showed a similar response to water supply to those of total sugar content, Brix, and glutamic acid content. Highest capsaicinoid content was observed in the drought treatment among the all treatments. However, Bosland and Votava (2002) recorded high capsaicinoid content in the fruit at a high rainfall site in New Mexico. The authors reported that the pungency of chili pepper fruit increased after water was applied by furrow irrigation. The present results are therefore incongruous. However, Estrada et al. (1999) reported similar results to the present findings in that capsaicinoid content was increased under drought stress. Sung et al. (2005) reported that the pungency under drought stress is stronger than that of the control and thus drought stress provides a good criterion for evaluation of hot pepper cultivars with high capsaicinoid content. Ruiz-Lau et al. (2011) reported that capsaicin and dihydrocapsaicin contents are increased in fruit of water-stressed plants of ‘Habanero’ pepper (*Capsicum chinense*) compared with those of control plants. Moreover, the contents are correlated with fruit age. These results are similar to those for capsaicinoid contents observed in the present experiment for *Capsicum annuum* cultivars. An additional possible reason for the increase in the capsaicinoid content of a whole fruit in response to drought stress is decrease in the placenta to pericarp ratio as a result of the reduction in fruit size. However, the capsaicinoid content of the placenta in fruit of chili pepper plants subjected to drought stress was higher than that of plants grown under excess water supply at the data in 2018. However, how the ratio of placenta and pericarp was effected to pungency of whole fruit was not clear, because I did not measured the ratio in this study, the reason of increase in capsaicinoid content in fruit by drought stress was presumably the capacity for capsaicinoid synthesis in the placenta was increased by the data from 2018.

The present study has clarified the relationships among fruit growth, water supply, and the taste components (sugars, glutamic acid, and capsaicinoids) of chili pepper fruit. However, it is necessary to further investigate the relationship between fruit yield, fruit size, fruit water content, leaf number, photosynthesis ability, and the contents of taste components to resolve how environmental factors affect the change in contents of taste components. In addition, it is necessary to elucidate of the genetic mechanism by conducting expression analysis of the genes involved in the synthesis and accumulation of taste components. These investigations was continued and discussed through the experiments in chapter VI and V.

Chapter III

Relationship between Salinity Stress and the Contents of Sugar and Capsaicinoids in the Fruit of Chili Peppers (*Capsicum* spp.)

Abstract

The amounts of the taste components sugars, glutamic acid, and capsaicinoids in chili pepper fruits (*Capsicum* spp.) change depending on environmental factors. The present study was conducted to evaluate the effects of salinity stress and harvesting days after flowering (DAF) on the sugar, glutamic acid, and capsaicinoid contents of the Chili pepper 'Manganji', 'Fushimiamanaga', 'Sapporo Oonaga Namban' (*C. annuum*), and 'Habanero' (*C. chinense*). Experiments were conducted in a greenhouse from April to October in 2018 and 2019. Three levels of salinity stress were applied: control (0.9 dS/m), additional (5 dS/m), and excessive (10 dS/m). Fruit was harvested at 20, 30, 40, and 50 DAF. The fruit weight (g) and contents of glucose, total sugar, and glutamic acid were measured using a portable spectrophotometer, and the capsaicinoid content was measured by high-performance liquid chromatography. The Brix and the contents of total sugar, glutamic acid, and capsaicinoids increased, while the glucose content decreased with the delay in harvesting. In the excessive salinity group, the glucose content tended to be lower, but the Brix and the contents of total sugar, glutamic acid, and capsaicinoids tended to be higher than those of the control group at each harvesting DAF in all Chili pepper.

Introduction

There are several domesticated species of Chili pepper in the world, and among them, *Capsicum annuum* L., *Capsicum frutescens* L., and *Capsicum chinense* Jacq., are the most widely used, and they include many common varieties. These Chili pepper are used around the world for their sensory properties, not only their pungency but their sweetness or umami also. Pungency, a commercially important attribute of Chili pepper, is conferred by the presence of chemicals from the characteristic capsaicinoids group. The substance that produces the heat sensation is known as capsaicin (8-methyl-*N*-vanillyl-trans-6-nonenamide). The most common capsaicinoids in Chili pepper are capsaicin and dihydrocapsaicin. The production of capsaicin in cultivated Chili pepper is influenced by genetic and environmental factors. Capsaicinoids are synthesized in the epidermal cells on the placental dissepiment (Sugiyama et al., 2006), and are composed of several different alkaloids, which vary in amount depending on the species (Rowland et al., 1983).

Environmental factors, especially the temperature, soil water conditions, and salinity, have been found to alter the plant chemistry. We clarified that drought stress significantly increased the amounts of total sugar, glutamic acid, and capsaicinoids in Chili pepper (Rathnayaka et al., 2021). On the other hand, for most plant species, high soil salinity levels create a stressful environment. These saline environments can be the result of oil and gas drilling, and farmland irrigation in the world's more arid zones (Ungar, 1991). In many areas of the world, soil salinization is an important concern for the sustainability of irrigated agriculture. High concentrations of salts in soil are responsible for large decreases in the yield of a cultivar of crops worldwide. It was estimated that approximately 20% (45 million ha) of irrigated land, producing one-third of the world's food, is salt-affected (Shrivastava and Kumar, 2015). Salinity inhibition of plant growth is the result of osmotic and ionic effects, and an ion imbalance due to decreased water and nutrient uptake (Greenway and Munns, 1980).

Salt tolerance is an important topic of study due to the loss of productive agricultural land to salinization. Salts can affect nutrient uptake in plants as well. In particular, nitrogen uptake can be reduced by high salinity, leading to reduced plant growth (Drake and Ungar, 1989). Although much research has been conducted to examine the effects of soil salinity on halophytic plant species (Ungar, 1991), more recent research has focused on salt tolerance among food crops, including Chili pepper (Van der Beek and Ltifi, 1991) and tomatoes (Li et al., 2011), two closely related members of the Solanaceae family.

However, to my knowledge, there have been few reports and little findings on the extent to which environmental factors influence the taste components, such as for pungency, and the contents of sugar and glutamic acid in Chili pepper. It has been shown that environmental factors, such as temperature, solar radiation, water stress, and soil nutrients, influence the contents of sugar and glutamic acid in tomatoes (Ortiz et al., 2007). Saito et al. (2006) reported that salinity stress can have a positive effect on the taste components in tomato fruits. However, in tropical countries, salinity stress is a major problem in plant cultivation. To the best of my knowledge, there have been no reports showing the effect of soil salinity on the contents of capsaicinoids and sugar in Chili pepper. Understanding whether salinity stress in soil affects Chili pepper fruit positively or negatively may enable the production of better-quality fruit in soil with accumulated salt. Therefore, the present study was conducted to determine the effects of different soil salinity stress conditions on the contents of sweet components and capsaicinoids in Chili pepper fruit.

Materials and Methods

Plant materials and experimental design

From April to October, 2018, an experiment was conducted in a greenhouse at the experimental farm (733 m a.s.l.) of the Education and Research Center of Alpine Field Science, Faculty of Agriculture, Shinshu University, located in Minamiminowa, Nagano, Japan. The experiment was repeated at the same location and during the same period in 2019. In 2018, two Japanese cultivar of Chili pepper, ‘Manganji’ and ‘Fushimiamanaga’, were used in the experiment. Both are non-pungent vegetable cultivar that originated in Kyoto Prefecture. Seeds of 'Manganji' were purchased from Noguchi Seeds (Saitama, Japan), and seeds of 'Fushimiamanaga' were purchased from Takii Seed Co., Ltd. (Kyoto, Japan). Also I used a more pungent cultivar called ‘Sapporo Oonaga Nanban’ (‘Sapporo’; Tsurushin Seed, Matsumoto, Japan), which is a local cultivar originating in Hokkaido, and a high pungent cultivar called ‘Habanero’ (Tsurushin Seed) as *C. chinense* (Fig. 1). Our preliminary experiments (Hatakeyama *et al*., 2017) found that ‘Manganji’ had the highest glucose and total sugar contents, and ‘Fushimiamanaga’ had the highest glutamic acid content among 39 tested strains. In addition, Hatakeyama *et al*. (2017) found that ‘Sapporo’ had the highest Brix value. Also, I selected ‘Habanero’ due to its high pungency and except cultivar from *Capsicum annuum*. The seeds were sown on April 5, 2018, and April 11, 2019. The seedlings were grown in a greenhouse in 75-mm plastic pots filled with a commercial potting medium (Nae-ichiban, Sumitomo Forestry Landscaping Co., Ltd., Tokyo, Japan).

Fig. 1. Fruit of Japanese chili pepper (*Capsicum annuum*) cultivars included in the study: (a) 'Manganji' (Noguchi Seeds); (b) 'Fushimiamanaga' (Takii Seed Co., Ltd.); (c) 'Sapporo Oonaga Nanban' (Tsurushin Seed); and (d) 'Habanero' (Tsurushin Seed).

Seedlings of approximately 150 mm in height were transplanted to plastic pots (diameter 18 cm, volume 1.8 L) filled with 1 kg of the same commercial potting medium on June 7, 2018, and June 16, 2019. Six individuals of each cultivar were used for each treatment.

During the cultivation period, a single application of slow-acting home gardening fertilizer (N:P:K, 10:10:10; Shizen Oyokagaku Co. Ltd., Nagoya, Aichi, Japan) was applied (on August 10, 2018, and August 15, 2019). The plastic pots filled with 1 kg of potting medium were filled to the brim with 130 mL of water. As treatments, I applied three salinity levels using sodium chloride (NaCl; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan); the levels were normal salinity control (C group; 0.9 dS/m (0.57 g/L)), additional salinity (A group; 5 dS/m (3.2 g/L)), and excessive salinity (E group; 10 dS/m (6.4 g/L)). Water was supplied with consideration of the daily temperature and weather. On sunny days and/or when the daily temperature exceeded 30°C, water was applied three times per day. On rainy or cloudy days and/or when the daily temperature was lower than 30°C, water was applied twice per day. During the period of the experiment, the average temperature of the greenhouse was 29.9°C (Maximum, 37.3°C; Minimum, 22.5°C) in 2018, and 29.3°C (Maximum, 35.3°C; Minimum, 23.2°C) in 2019. Throughout the experiment, other management practices were applied equally to all plants in the same greenhouse.

In both years, the stress treatment was applied starting 1 week after the seedlings were transplanted. All flowers that had flowered before the stress treatment was applied were removed. During the stress treatment, each flower was tagged at the end of anthesis to ensure that fruit could be harvested at 20, 30, 40, and 50 DAF. The harvested fruit was stored at −80°C until analysis.

Fruit harvested for each salinity treatment and at each harvesting date were used for the analysis of the fruit weight, contents of glucose, total sugar (glucose and fructose), glutamic acid, and capsaicinoids, as well as the Brix percentage. Fruit from each individual was harvested at 20, 30, 40, and 50 DAF. Three samples, each comprising 50 g of fruit (approximately 1 to 6 fruit), were chosen randomly for the analysis of taste components and six individuals from each treatments used to collect weight of the fruits. This sampling amount was used, because the extract from 50 g of fruit tissue was required for spectrophotometric analysis. A randomized complete block design was used for the experiments.

During the experimental period in 2018, some plants in the excessive salinity stress treatment group and the additional salinity stress treatment group exhibited leaf burn, and most of their leaves fell off. Because of this unfortunate occurrence, I stopped inducing salinity stress on the 29th of June, 2018. Subsequently, the damaged plants recovered within 2 weeks, and then continued all treatments again on the 13th of August, 2018.

Solution preparation for analysis of sugar and glutamic acid contents

Extracts for sugar and glutamic acid analyses were prepared from a known amount of fruit tissue ground using a grinder (YMB-400, Yamazen, Osaka, Japan) and filtered through a 125-mm filter paper (ADVANTEC, Tokyo, Japan). Extracts were prepared for the quantitative analysis of glucose (Strips- Reflctoquant® Glucose-Test, Merck KGaA, Darmstadt, Germany), total sugar (Strips- Reflctoquant® Total Sugar-Test (glucose and fructose), Merck KGaA, Darmstadt, Germany), and glutamic acid (Strips- Agrocheck Glutamic acid test, Kanto Chemical, Co., Inc., Tokyo, Japan) using a digital portable spectrophotometer (RQflex plus 10, Merck, Darmstadt, Germany). According to Nonaka et al. (2012), the RQflex spectrophotometer and capillary electrophoresis methods (Horie, 2009) yield similar results for the contents of glucose, total sugar, and glutamic acid in Chili pepper fruit.

Solution preparation for analysis of Brix

Brix is primarily a measure of the sugar concentration in a solution. Extracts were prepared from fruit tissue ground using a grinder (YMB-400, Yamazen) and filtered through a 125-mm filter paper (ADVANTEC). Extracts were used directly to measure the Brix value with a digital portable refractometer (Pen-J, Atago Co., Ltd., Tokyo, Japan).

Capsaicinoids analysis - HPLC apparatus and analysis conditions

The high-performance liquid chromatography analysis conditions were as follows: liquid chromatography column (50 × 3.0 mm; Shimadzu Corporation, Kyoto, Japan); column temperature, 40°C; mobile phase, 70% methanol; flow rate, 1 mL/min; and absorbance at a wavelength of 280 nm. To examine the effectiveness of the analysis conditions, capsaicin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a standard. Standard capsaicin solutions of 62.5, 125, 250, and 500 µg/mL (Othman et al., 2011) were analyzed, and a calibration curve was prepared. Sample solutions were prepared by using 24-h freeze-dried Chili pepper fruit; capsaicinoids were extracted by adding ground Chili pepper powder (2 mg) to methanol (20 mL) and incubating for 1 h at 40°C.

Results

Fruit fresh weight (g)

The fresh fruit weight (g) of Chili pepper fruit showed a gradual increase with increasing harvesting DAF up to 50 DAF in each treatment group of all cultivar.

In 2018, the fruit weight of ‘Manganji’ harvested at 20 DAF was significantly higher in the normal salinity control (which is regarded as the “C group” in this study; plants exposed to excessive salinity and additional salinity are “E group” and “A group, respectively; the same hereinafter) than in the E group (Fig. 2-M1). In 2019, the fruit weight of ‘Manganji’ was significantly higher in the C group, followed by the A group and E group at 40 and 50 DAF (Fig. 2-M2). In ‘Manganji’ fruit harvested at 20 DAF in 2019, a significant difference was observed between the C and E groups. In ‘Fushimiamanaga’ fruit, the fruit weight was significantly lower in the E group than in the C group at 30, 40, and 50 DAF in 2018 (Fig. 2-F1). In 2019, a significant difference was observed between the fruit of the C and E groups harvested at all DAF (Fig. 2-F2). The fruit of ‘Sapporo’ showed similar trends, with significant differences seen in the fruit weight of the C and E groups when harvested at 20 and 30 DAF in 2018 (Fig. 2-S1) and 2019 (Fig. 2-S2). In ‘Habanero’ in 2018, a significantly higher fruit weight was observed in the fruit of the C group when compared to the fruit of the other groups when harvested at 40 and 50 DAF, and a significant difference was observed between the fruit of the C and E groups when harvested at 20 and 30 DAF (Fig. 2-H1). In 2019, a significantly higher fruit weight was observed in the fruit of the C group when compared to the fruit of the other groups harvested at 30 and 50 DAF, and between the fruit of the C and E groups when harvested at 20 and 40 DAF (Fig. 2-H2).

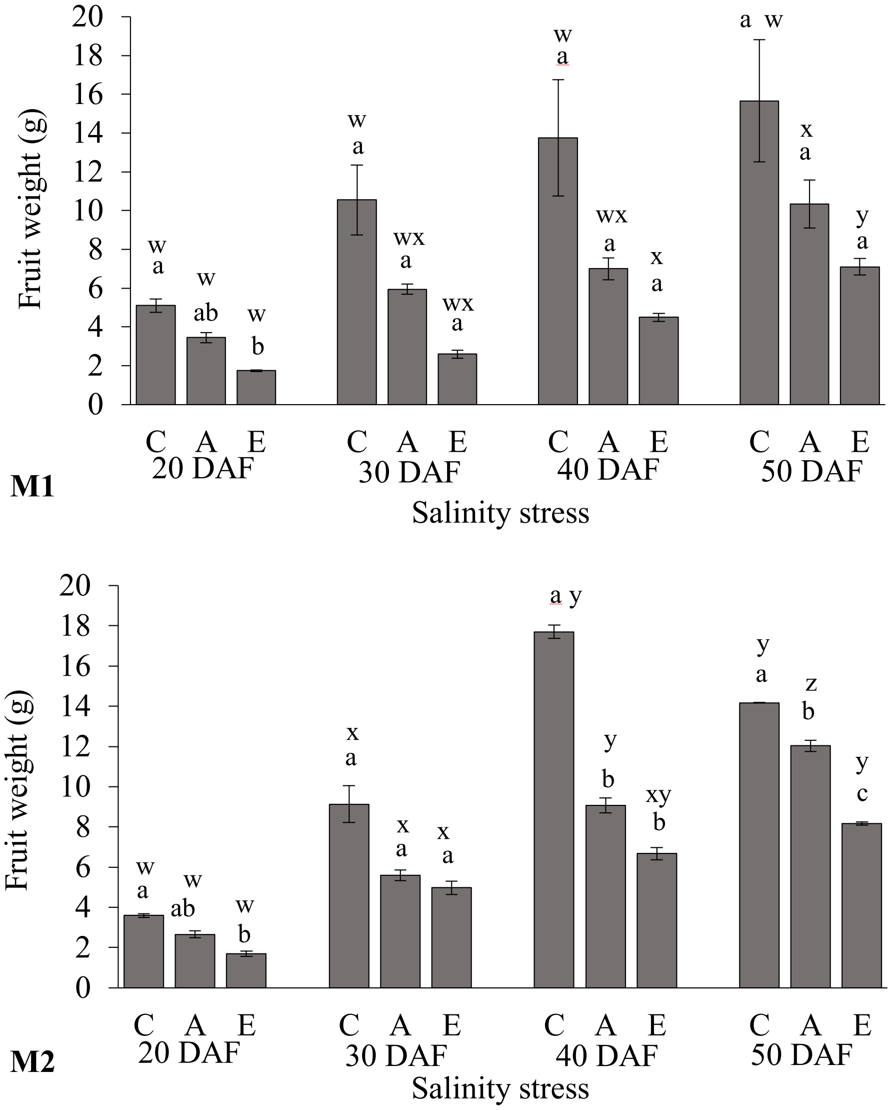


Fig. 2-M. Fruit weight (g [FW]) per fruit basis, at four stages of fruit maturation in the chili pepper 'Manganji' (M). Graphs M1 show the results of the year 2018, and M2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

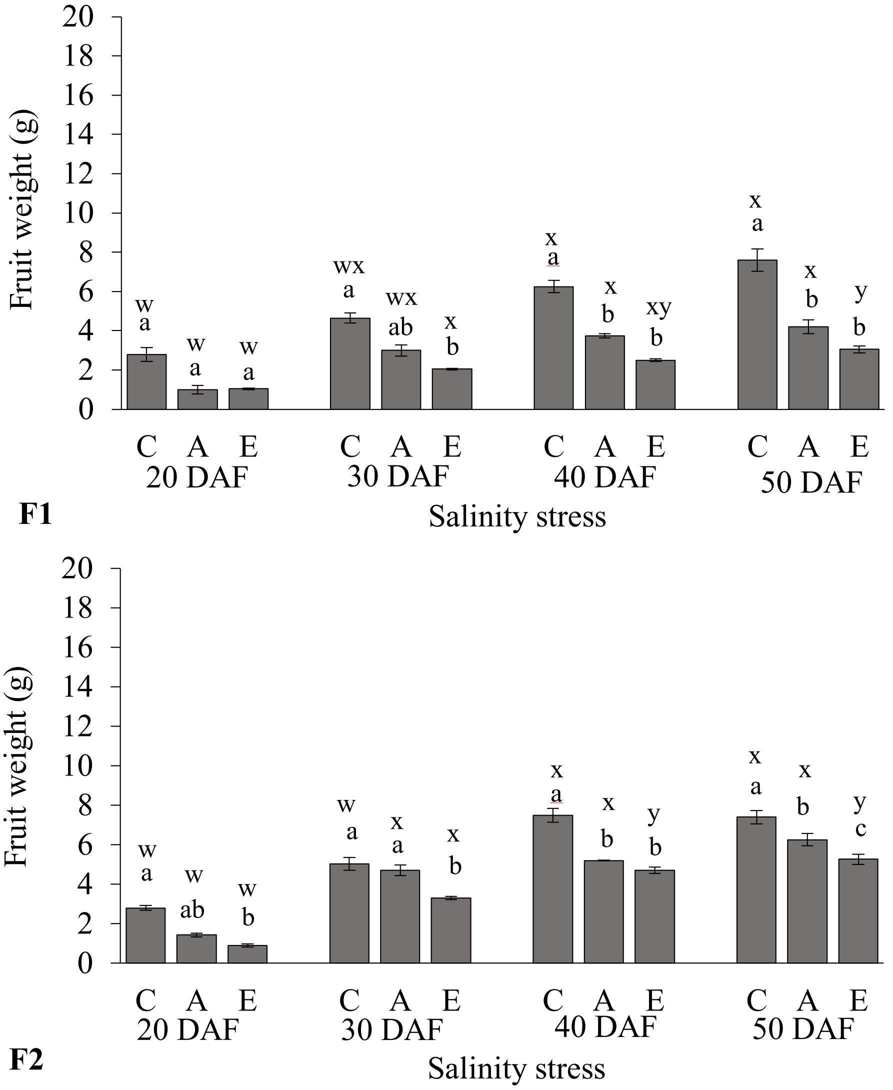


Fig. 2-F. Fruit weight (g [FW]) per fruit basis, at four stages of fruit maturation in the chili pepper ‘Fushimiamanaga’ (F). Graphs F1 show the results of the year 2018, and F2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

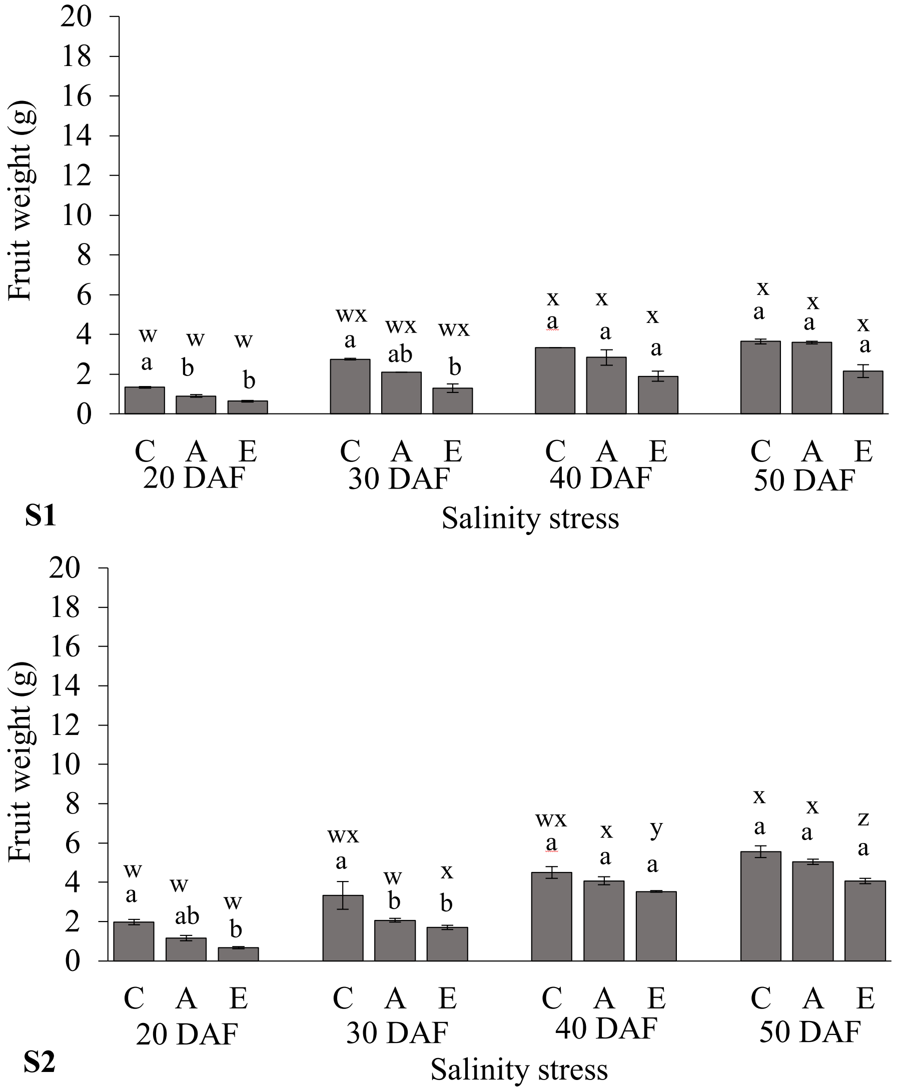


Fig. 2-S. Fruit weight (g [FW]) per fruit basis, at four stages of fruit maturation in the chili pepper ‘Sapporo’ (S). Graphs S1 show the results of the year 2018, and S2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

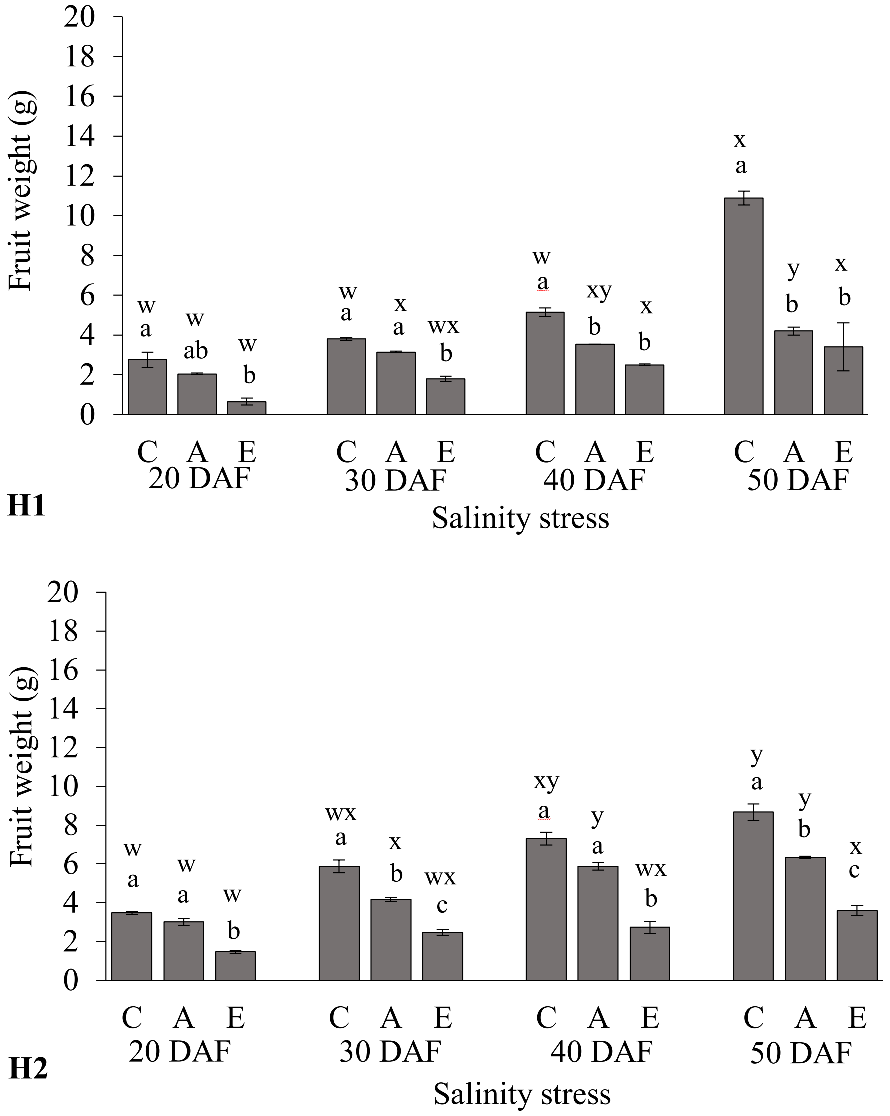


Fig. 2-H. Fruit weight (g [FW]) per fruit basis, at four stages of fruit maturation in the chili pepper 'Habanero' (H). Graphs H1 show the results of the year 2018, and H2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

Glucose content

The glucose content, expressed on a fresh weight (FW) basis, of Chili pepper fruit showed a gradual decrease with increasing harvesting DAF up to 50 DAF in each of the treatment groups of all cultivar. Among all of the cultivar, the C group of ‘Fushimiamanaga’ harvested at 20 DAF in 2019 showed the highest glucose content, and the lowest glucose content was seen in the E group of ‘Sapporo’ harvested at 50 DAF in 2018.

In 2018, the glucose content of ‘Manganji’ was significantly higher in fruit harvested at 20, 30, and 40 DAF in the C group than in the other groups, and it was also significantly higher in the C group than in the E group at 50 DAF (Fig. 3-M1). In 2019, the glucose content of ‘Manganji’ was significantly higher in the C group, followed by the A group and E group at 20 and 40 DAF (Fig. 3-M2). In ‘Manganji’ fruit harvested at 30 and 50 DAF in 2019, a significantly higher glucose content was observed in the C group than in the E group. In ‘Fushimiamanaga’ fruit, the glucose content was significantly lower in the E group than in the C group at 20, 40, and 50 DAF in 2018 (Fig. 3-F1). In both years, a significant difference was seen between all treatment groups in the fruit harvested at 30 DAF. In ‘Fushimiamanaga’ fruit harvested at 20 and 40 DAF in 2019, the glucose content of the E group was significantly lower than that of the C group (Fig. 3-F2). The fruit of ‘Sapporo’ showed similar trends, with a significantly higher glucose content in the C group than in the other groups in fruit harvested at 40 DAF in 2018 (Fig. 3-S1). In addition, the glucose content of the E group was lower than that of the C group at 20, 30, and 50 DAF. In ‘Sapporo’ fruit harvested at 40 and 50 DAF in 2019, a significantly lower glucose content was seen in the E group than in the C group (Fig. 3-S2). In ‘Habanero’ in 2018, a significantly higher glucose content was observed in the fruit of the C group than in the fruit of the other groups when harvested at 20 DAF, and a significant difference was observed between the fruit of the C and E groups when harvested at 30 DAF (Fig. 3-H1). In 2019, a significantly higher glucose content was observed in the fruit of the C group when harvested at 20, 30, and 50 DAF than in the fruit of the other groups, and significantly higher glucose content was observed in the fruit of the C than E group when harvested at 40 DAF (Fig. 3-H2).

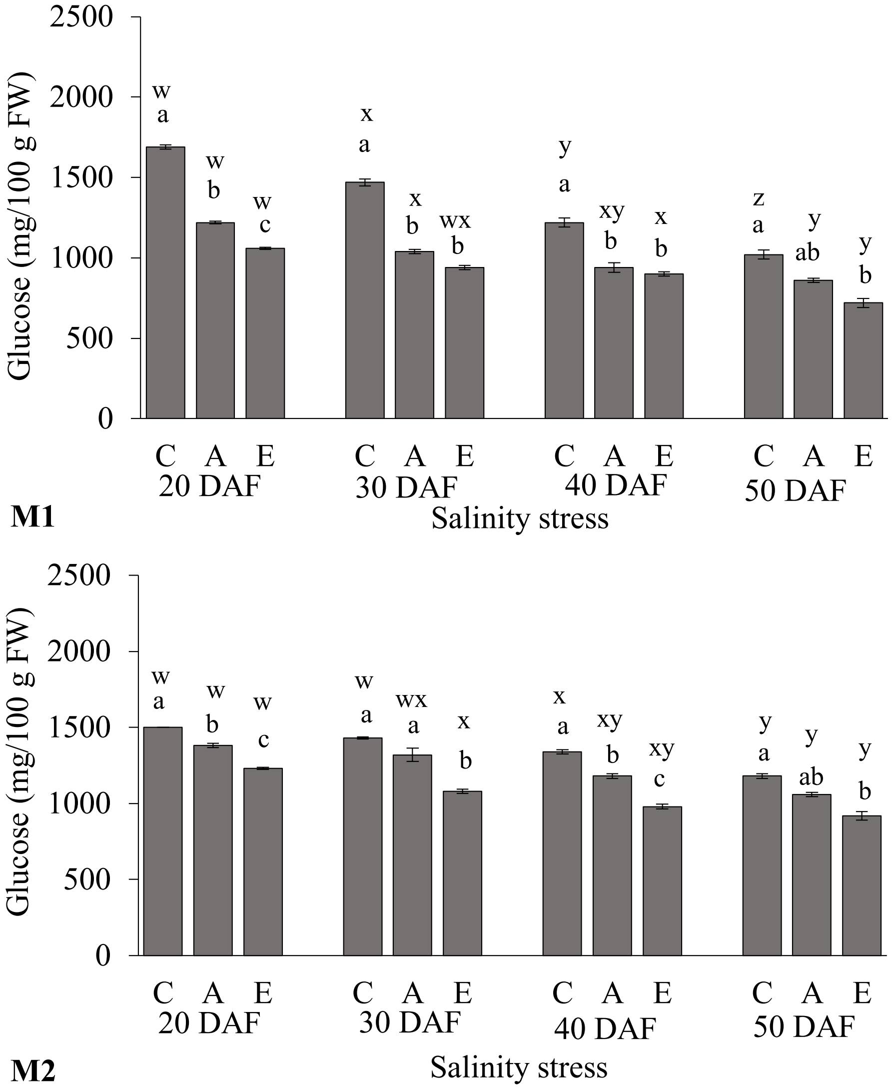


Fig. 3-M. Glucose content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper ‘Manganji’ (M). Graphs M1 show the results of the year 2018, and M2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, *P* < 0.05). Error bars indicate the standard error.

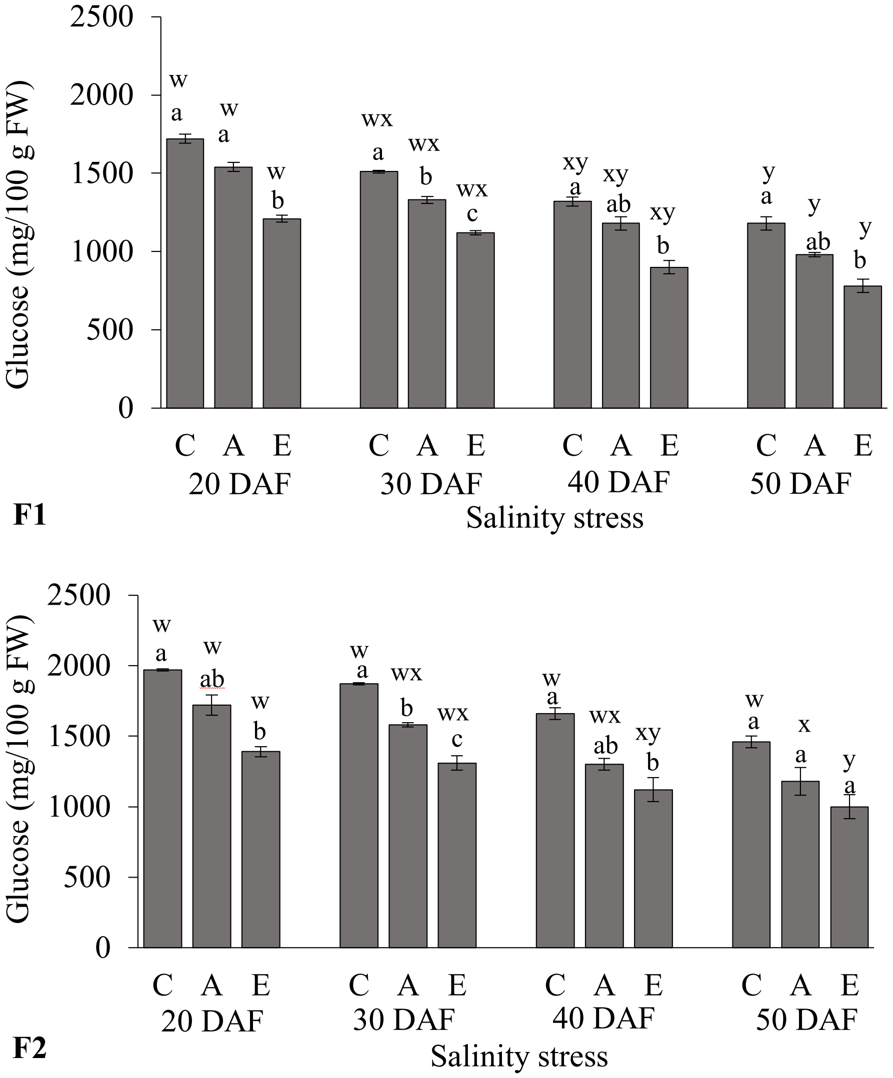


Fig. 3-F. Glucose content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper 'Fushimiamanaga' (F). Graphs F1 show the results of the year 2018, and F2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, *P* < 0.05). Error bars indicate the standard error.

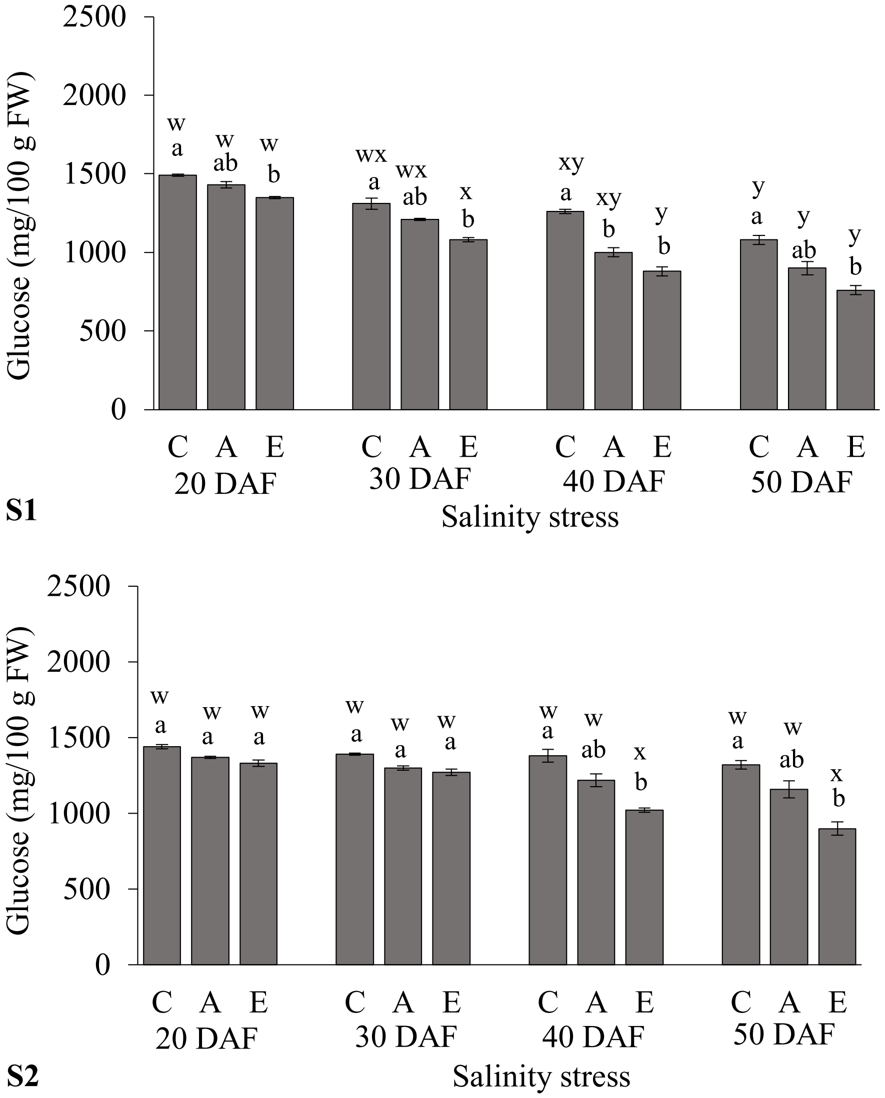


Fig. 3-S. Glucose content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper ‘Sapporo Oonaga Nanban’ (S). Graphs S1 show the results of the year 2018, and S2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

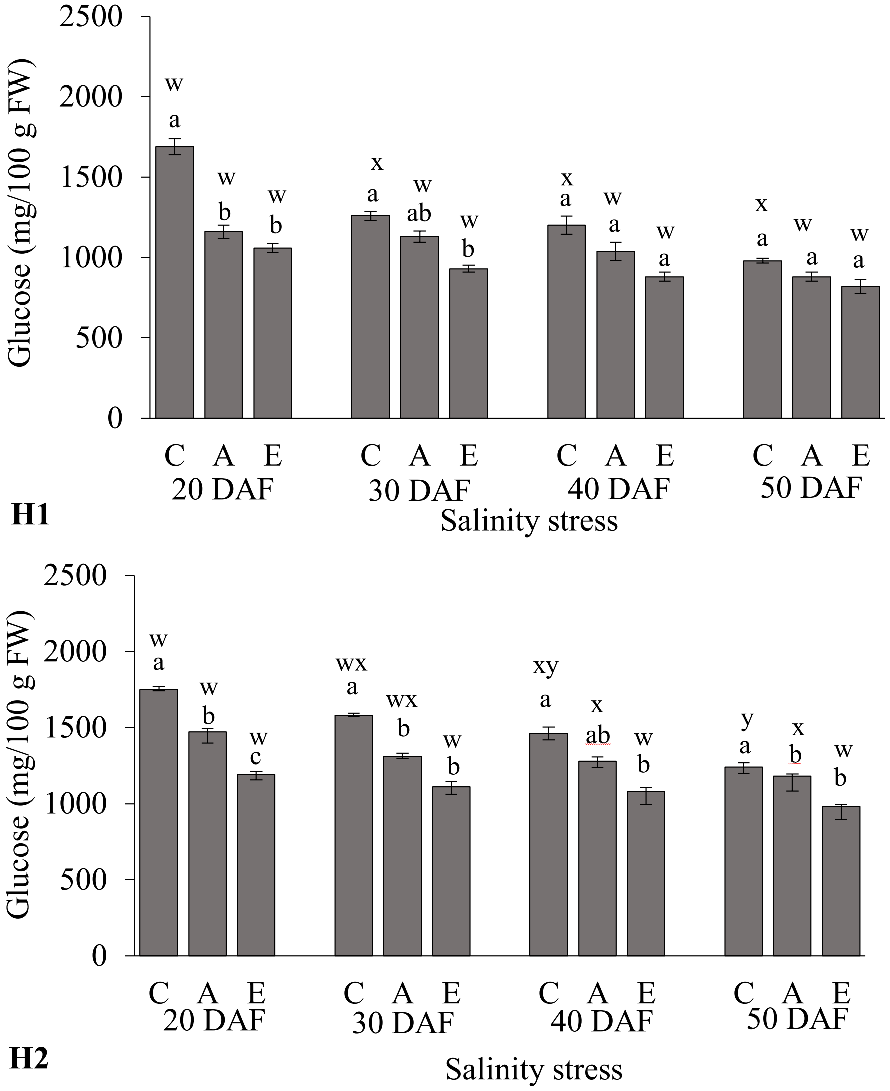


Fig. 3-H. Glucose content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper ‘Habanero’ (H). Graphs H1 show the results of the year 2018, and H2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

Total sugar content

The total sugar content, expressed on a FW basis, increased with the progression of fruit maturation in all cultivar. The highest total sugar content was observed in the fruit of the E group harvested at 50 DAF. The content of total sugar was increased in the E group when compared to the C group. Interestingly, this pattern was opposite to the trend seen for the glucose content in the same cultivar. Among all of the cultivar, the E group of ‘Manganji’ harvested at 50 DAF in 2019 showed the highest total sugar content, and the lowest total sugar content was seen in the C group of ‘Sapporo’ harvested at 20 DAF in 2019.

Fruit of ‘Manganji’ showed a significantly higher total sugar content in the E group than in the C group at all harvesting DAF in 2018 (Fig. 4-M1) and in 2019 (Fig. 4-M2). The total sugar content of ‘Fushimiamanaga’ fruit showed identical trends. The total sugar content of ‘Fushimiamanaga’ was significantly higher in the E group than in the other groups at 30 and 50 DAF in 2018 (Fig. 4-F1) and at 50 DAF in 2019 (Fig. 4-F2). In addition, a significant difference between the C and E groups was observed in the fruit harvested at 20 and 40 DAF in 2019, with higher values seen in the E group. In 2018, among the fruit of ‘Sapporo’ harvested at 20, 30, and 50 DAF, a significantly higher total sugar content was seen in the E group than in the other groups (Fig. 4-S1); this was also seen at all DAF in 2019 (Fig. 4-S2). The fruit of ‘Habanero’ harvested at 40 and 50 DAF in the E group had a significantly higher total sugar content than those in the other groups in 2018, and in the same year, a significant difference between the E and C groups was seen in the fruit harvested at 20 and 30 DAF (Fig. 4-H1). Fruit of ‘Habanero’ showed a significantly higher total sugar content in the E group, followed by the A group and C group at all DAF in 2019 (Fig. 4-H2).

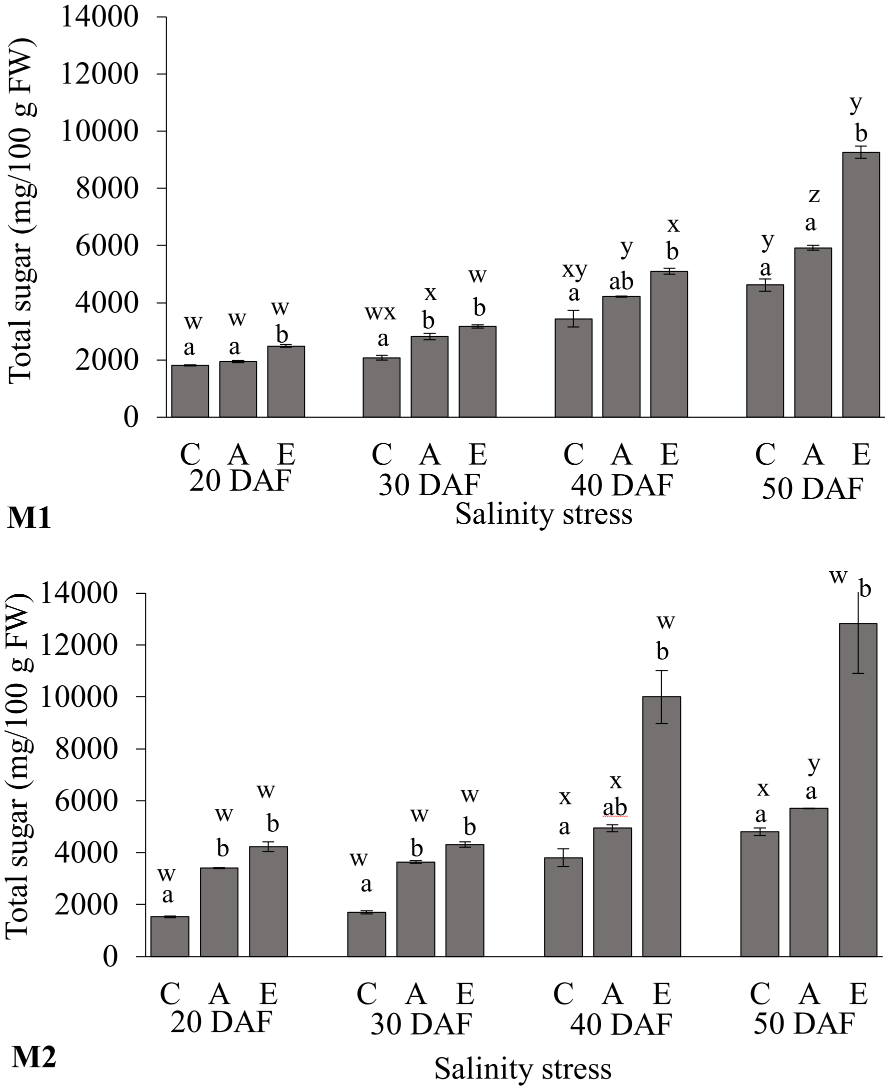


Fig. 4-M. Total sugar content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper ‘Manganji’ (M). Graphs M1 show the results of the year 2018, and M2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

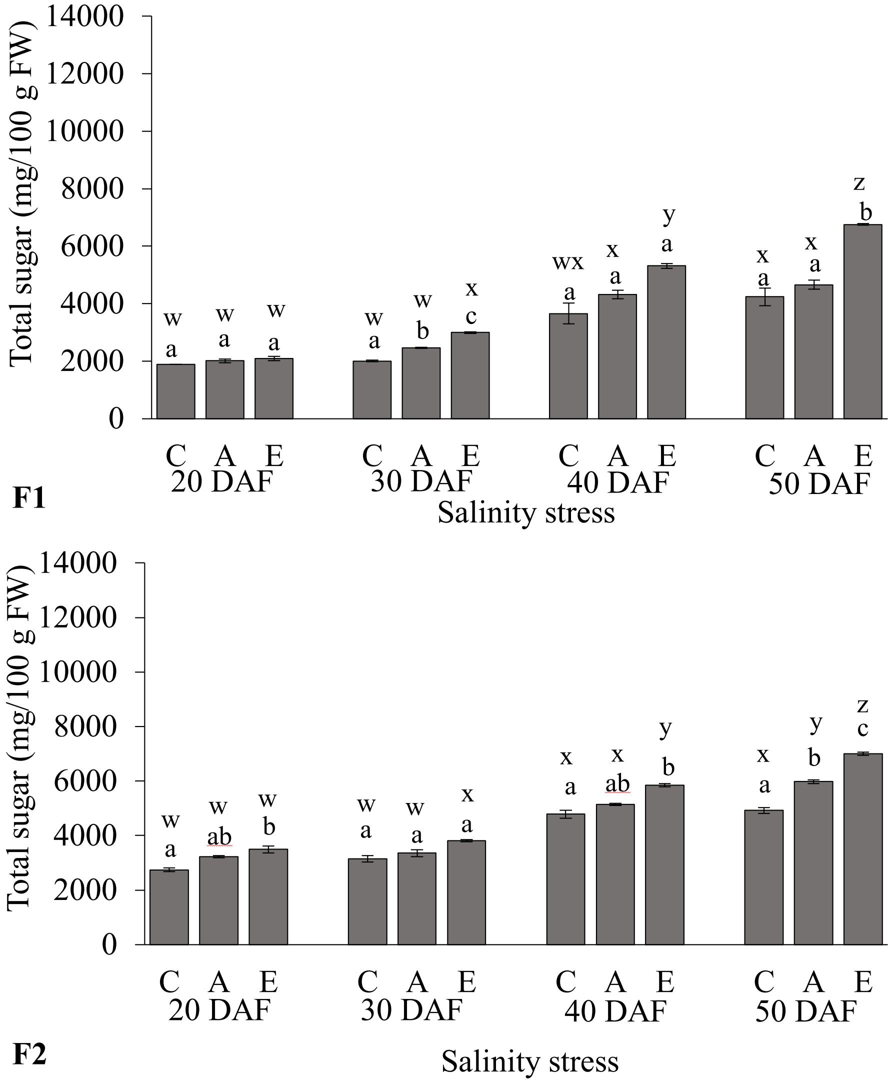


Fig. 4-F. Total sugar content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper ‘Fushimiamanaga’ (F). Graphs F1 show the results of the year 2018, and F2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, *P* < 0.05). Error bars indicate the standard error.

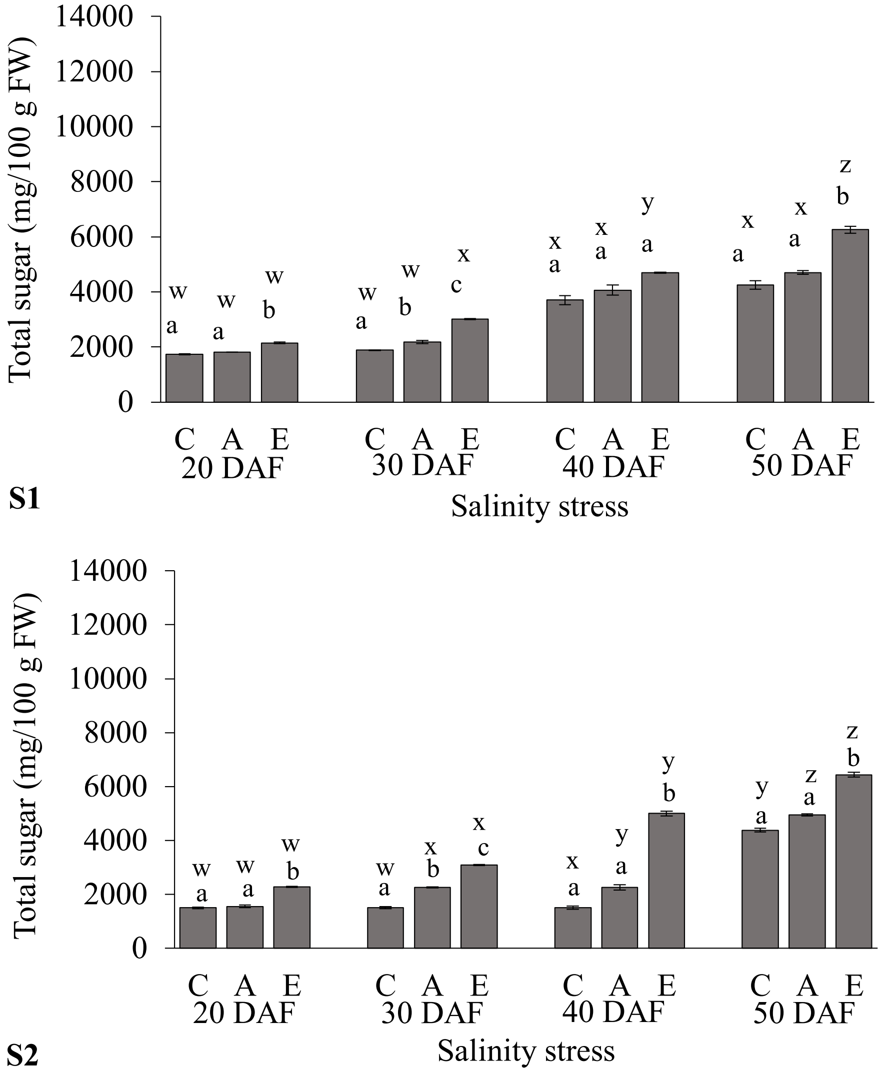


Fig. 4-S. Total sugar content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper ‘Sapporo’ (S). Graphs S1 show the results of the year 2018, and S2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

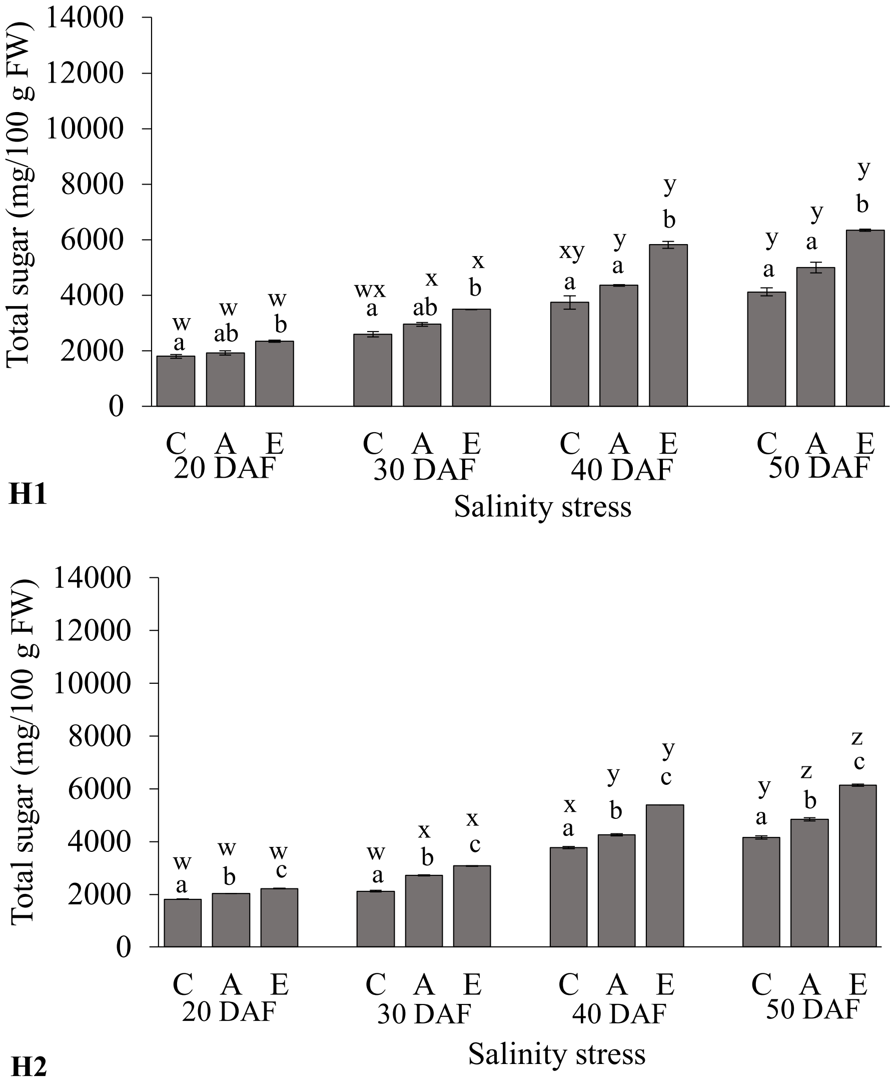


Fig. 4-H. Total sugar content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper 'Habanero' (H). Graphs H1 show the results of the year 2018, and H2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, *P* < 0.05). Error bars indicate the standard error.

Brix

The Brix percentage in the fruit of all four cultivar tended to increase with increasing salinity in the soil and with increasing delay in harvesting. The highest Brix percentage was observed in the E group at an advanced stage of maturation (at 50 DAF). Among all of the cultivar, the E group of 'Fushimiamanaga' harvested at 50 DAF in 2018 showed the highest Brix percentage, and the lowest Brix percentage was seen in the C group of 'Fushimiamanaga' harvested at 20 DAF in 2019.

Among 'Manganji' fruit, a significantly higher Brix percentage was observed in the E group than in the C group at 30 and 50 DAF in 2018 (Fig. 5-M1), and at 50 DAF in 2019. In addition, a significantly higher Brix percentage was observed in the fruit of the E group than in the other groups when harvested at 20 and 30 DAF in 2019 (Fig. 5-M2). The differences in Brix were similar in the other three cultivar. In 'Fushimiamanaga', the Brix of fruit harvested at 40 and 50 DAF differed significantly between the E and C groups in 2018, with a higher percentage seen in the E group (Fig. 5-F1). In 2019, the Brix of fruit harvested at 20 and 30 DAF was significantly higher in the E group than in the other groups. Also, when harvested at 40 and 50 DAF in 2019, the Brix of fruit was significantly higher in the E group than in the C group (Fig. 5-F2). In 'Sapporo', fruit harvested at all DAF in 2018 showed a significantly higher Brix in the E group than in the C group (Fig. 5-S1); this was also seen for fruit harvested at 20, 30, and 40 DAF in 2019. In the same year, when harvested at 50 DAF, the Brix percentage differed significantly between all treatment groups (Fig. 5-S2). Among the fruit of 'Habanero', a significantly higher Brix was observed in the E group than in the C group at 30 DAF in 2018 (Fig. 5-H1). The Brix of 'Habanero' was significantly higher in the E group, followed by the A group and C group at all DAF in 2019 (Fig. 5-H2).

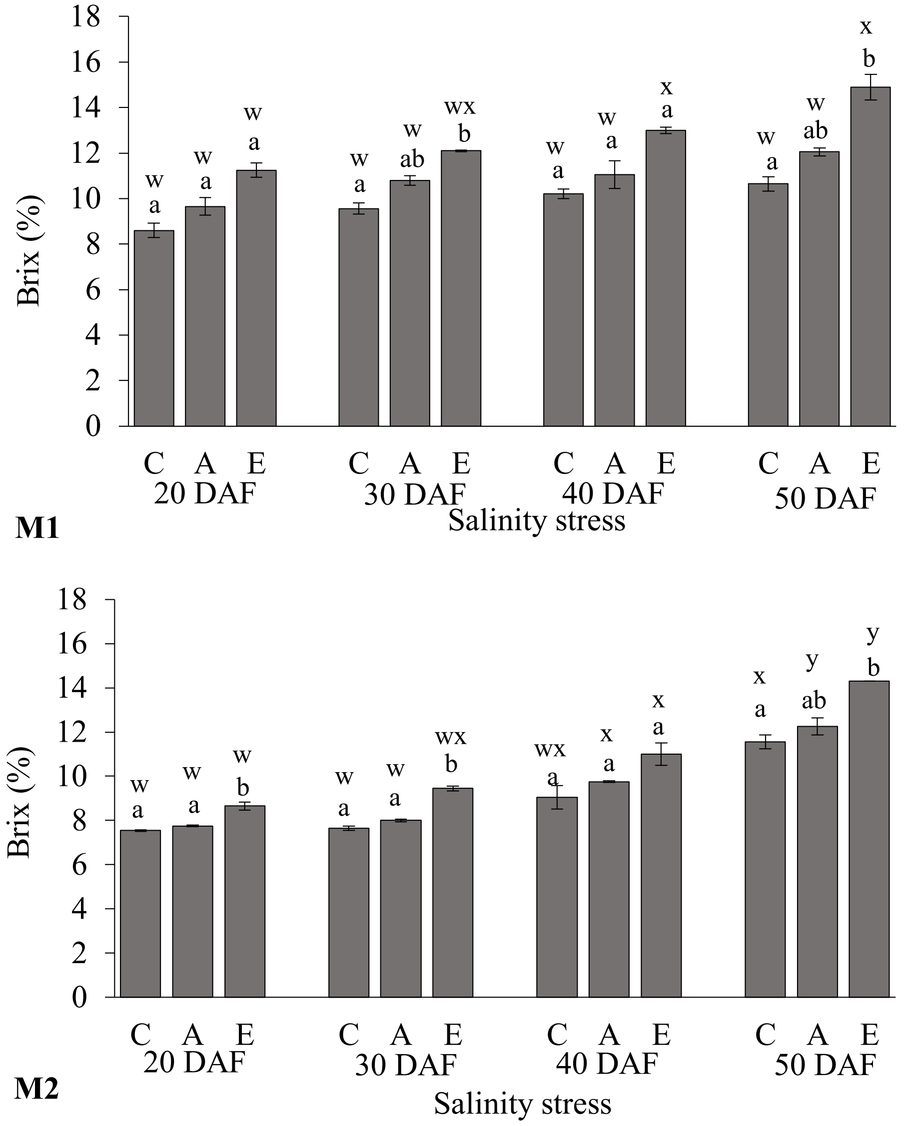


Fig. 5-M. Brix at four stages of fruit maturation in the chili pepper 'Manganji' (M). Graphs M1 show the results of the year 2018, and M2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

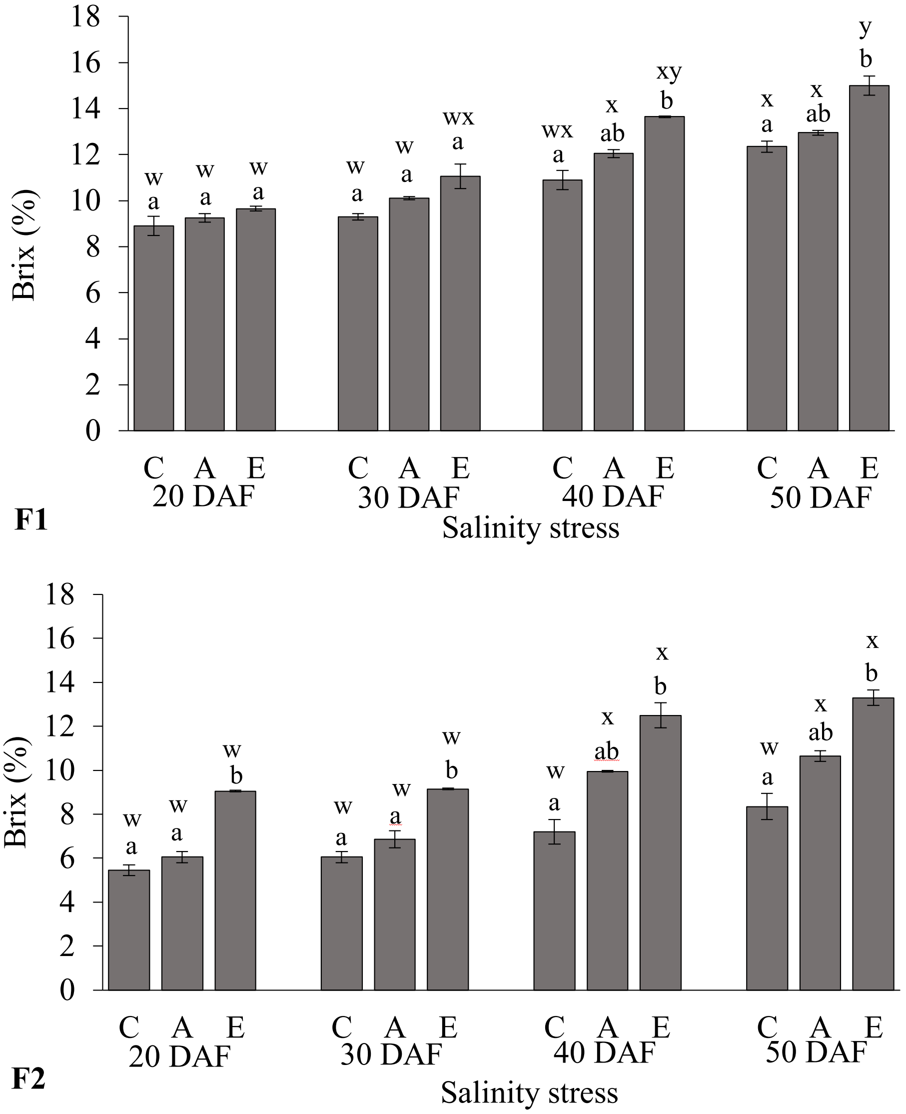


Fig. 5-F. Brix at four stages of fruit maturation in the chili pepper ‘Fushimiamanaga’ (F). Graphs F1 show the results of the year 2018, and F2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

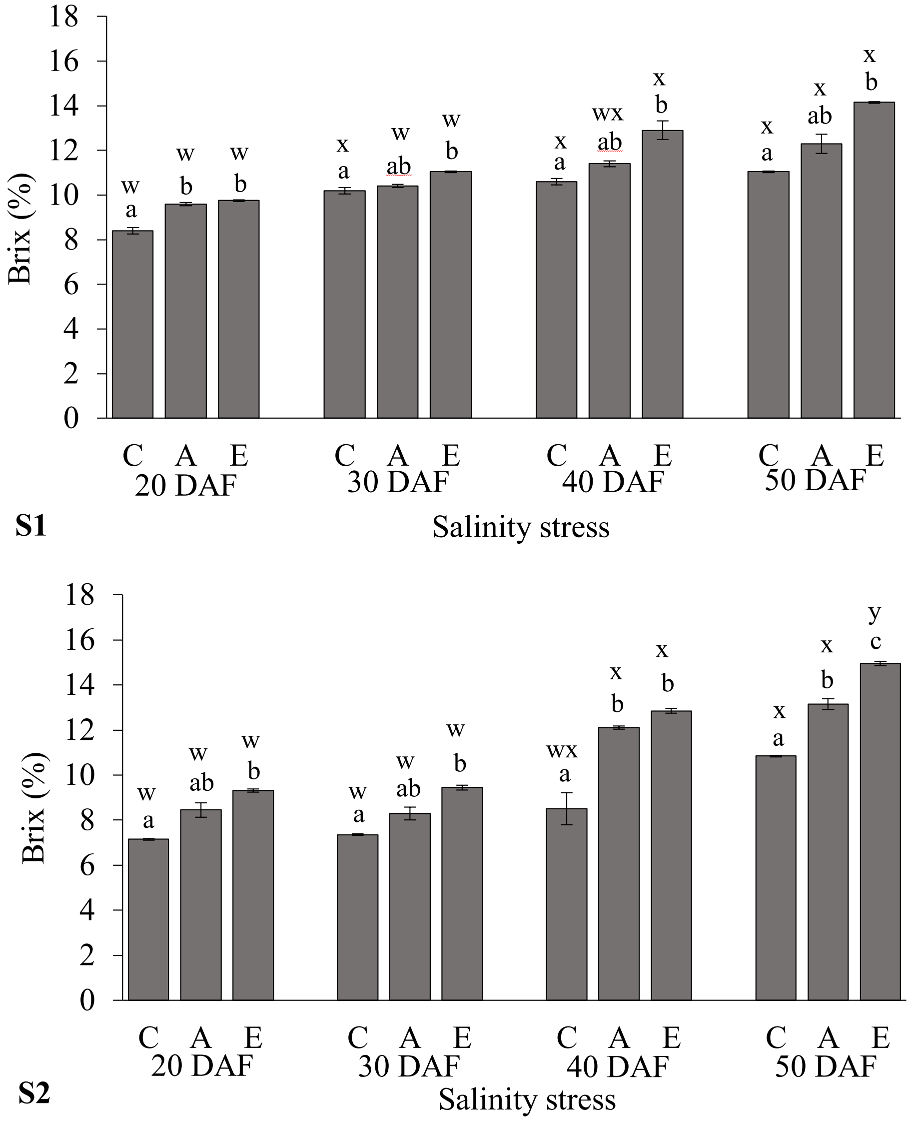


Fig. 5-S. Brix at four stages of fruit maturation in the chili pepper ‘Sapporo’ (S). Graphs S1 show the results of the year 2018, and S2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a b, and c, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error.

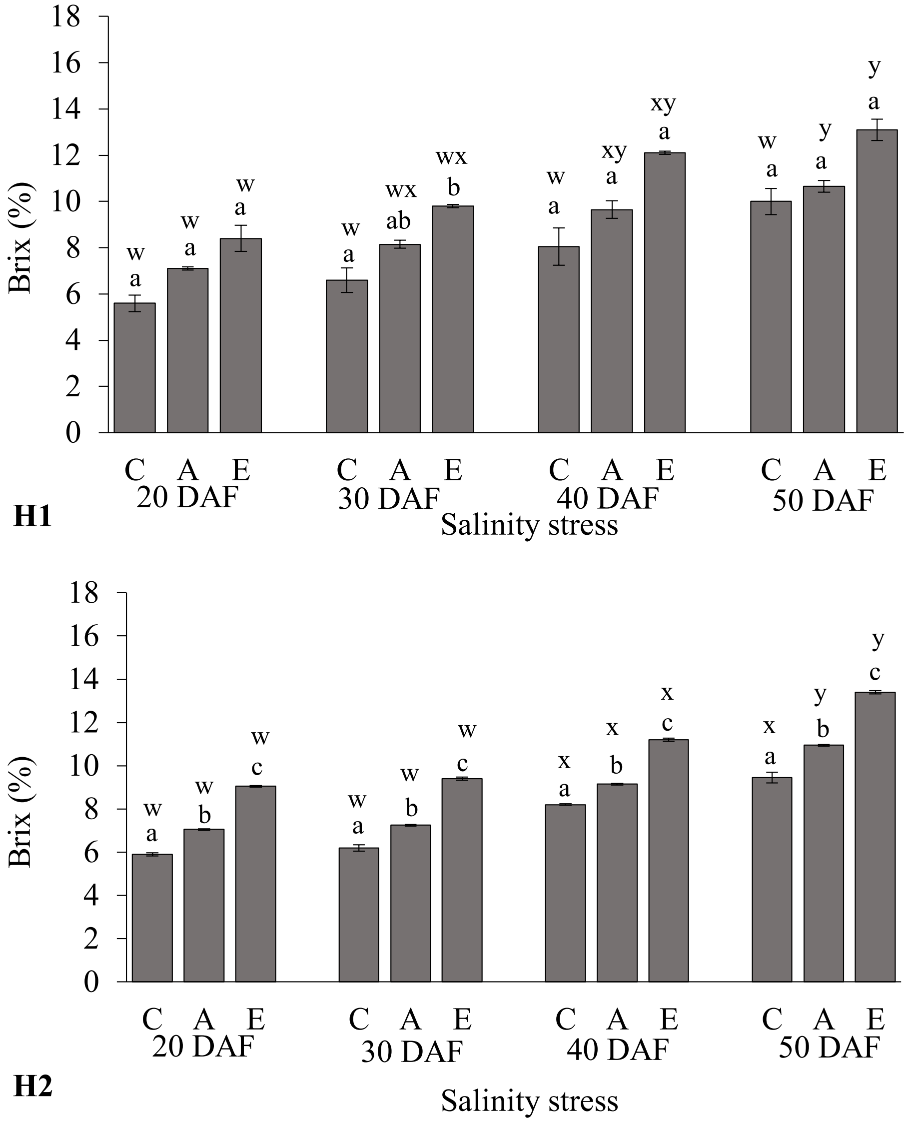
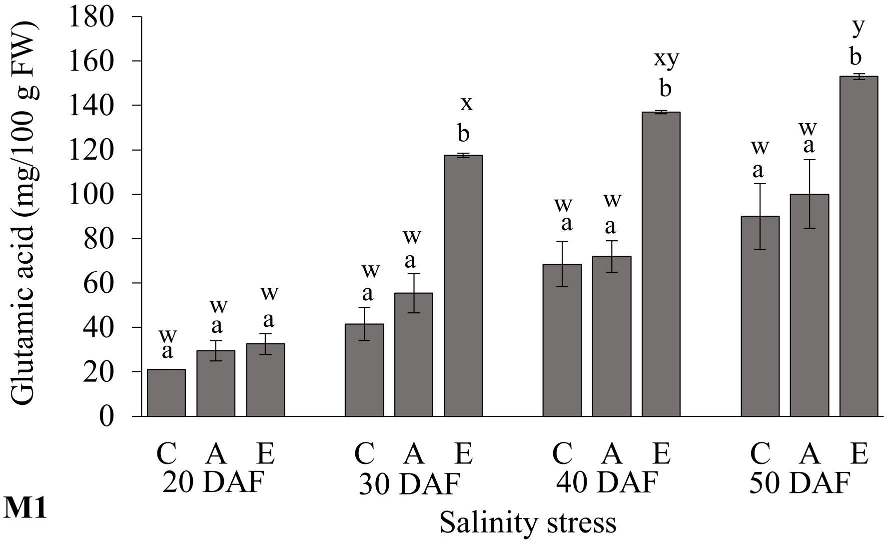


Fig. 5-H. Brix at four stages of fruit maturation in the chili pepper 'Habanero' (H). Graphs H1 show the results of the year 2018, and H2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a b, and c, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

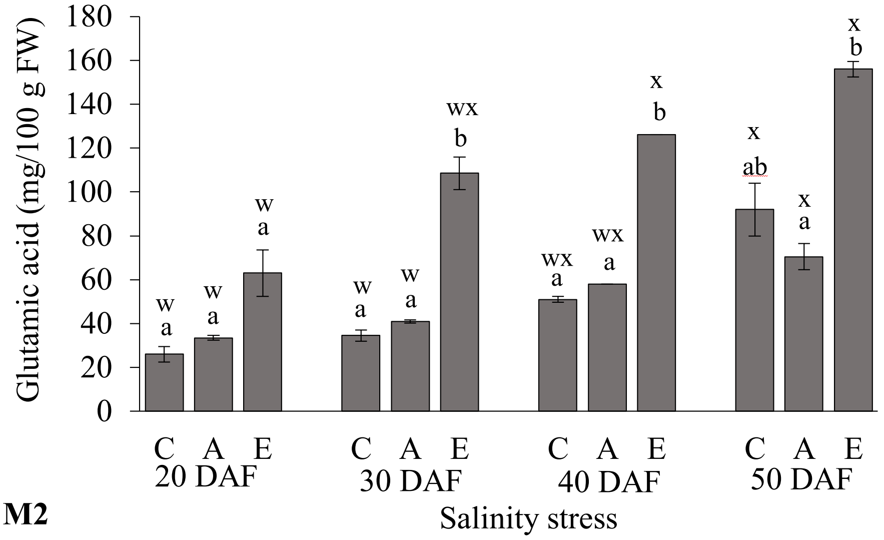
Glutamic acid content

Glutamic acid content, expressed on a FW basis, increased in each cultivar with increasing delay in harvesting. In addition, all cultivar showed higher contents of glutamic acid in the E group than in the other groups. Among all of the cultivar, the E group of 'Manganji' harvested at 50 DAF in 2019 showed the highest glutamic acid content, and the lowest glutamic acid content was seen in the C group of 'Habanero' harvested at 20 DAF in 2018.

In 'Manganji', a significantly higher glutamic acid content was observed in the E group than in the other groups at 30, 40, and 50 DAF in 2018 (Fig. 6-M1), and at 30 and 40 DAF in 2019 (Fig. 6-M2). In 2018, a significantly higher glutamic acid content was observed in 'Fushimiamanaga' in the E group than in the other groups, except for fruit harvested at 20 DAF (Fig. 6-F1). In 'Fushimiamanaga', the glutamic acid content was significantly higher in fruit of the E group when compared to the C group at 30, 40, and 50 DAF in 2019 (Fig. 6-F2). In 'Sapporo' fruit, the glutamic acid content was significantly higher in the E group than in the other groups at 40 and 50 DAF in 2018 (Fig. 6-S1), and at all DAF in 2019 (Fig. 6-S2). The glutamic acid content in 'Habanero' fruit was significantly higher in the E group, followed by the A group and C group at all DAF in 2018 (Fig. 6-H1), and at 20 and 40 DAF in 2019. In addition, significant differences between the E group and the other groups were observed in 'Habanero' fruit at 30 and 50 DAF (Fig. 6-H2).



M1



M2

Fig. 6-M. Glutamic acid content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper 'Manganji' (M). Graphs M1 show the results of the year 2018, and M2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, *P* < 0.05). Error bars indicate the standard error.

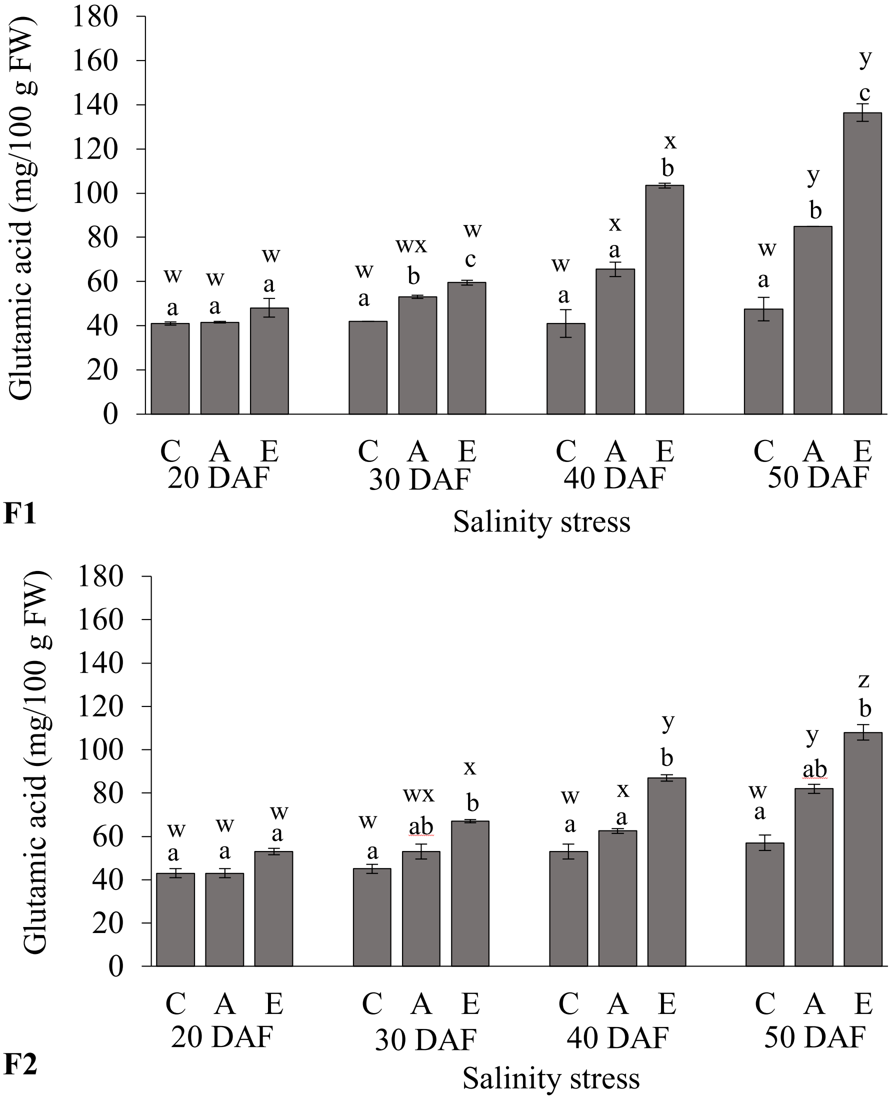


Fig. 6-F. Glutamic acid content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper 'Fushimiamanaga' (F). Graphs F1 show the results of the year 2018, and F2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

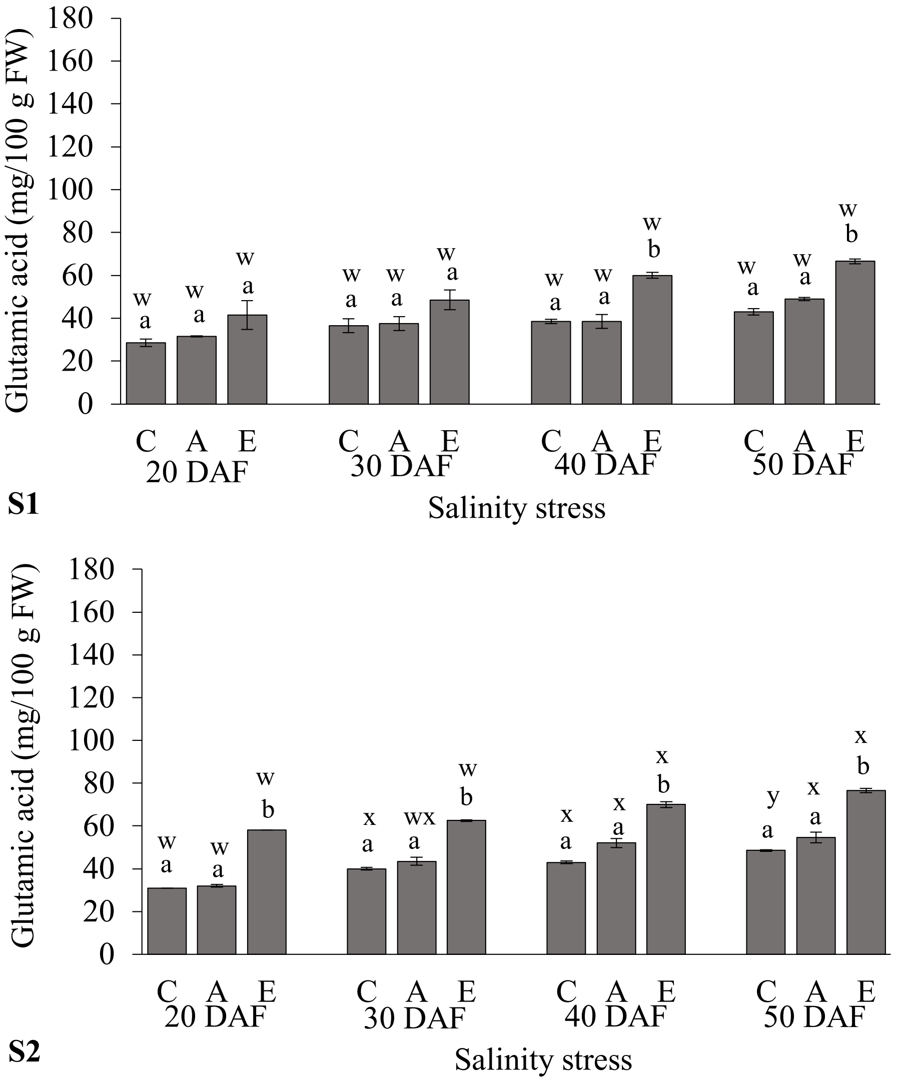


Fig. 6-S. Glutamic acid content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper 'Sapporo' (S). Graphs S1 show the results of the year 2018, and S2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a and b, above the bars for the same DAF, and different lowercase letters, w, x, and y, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

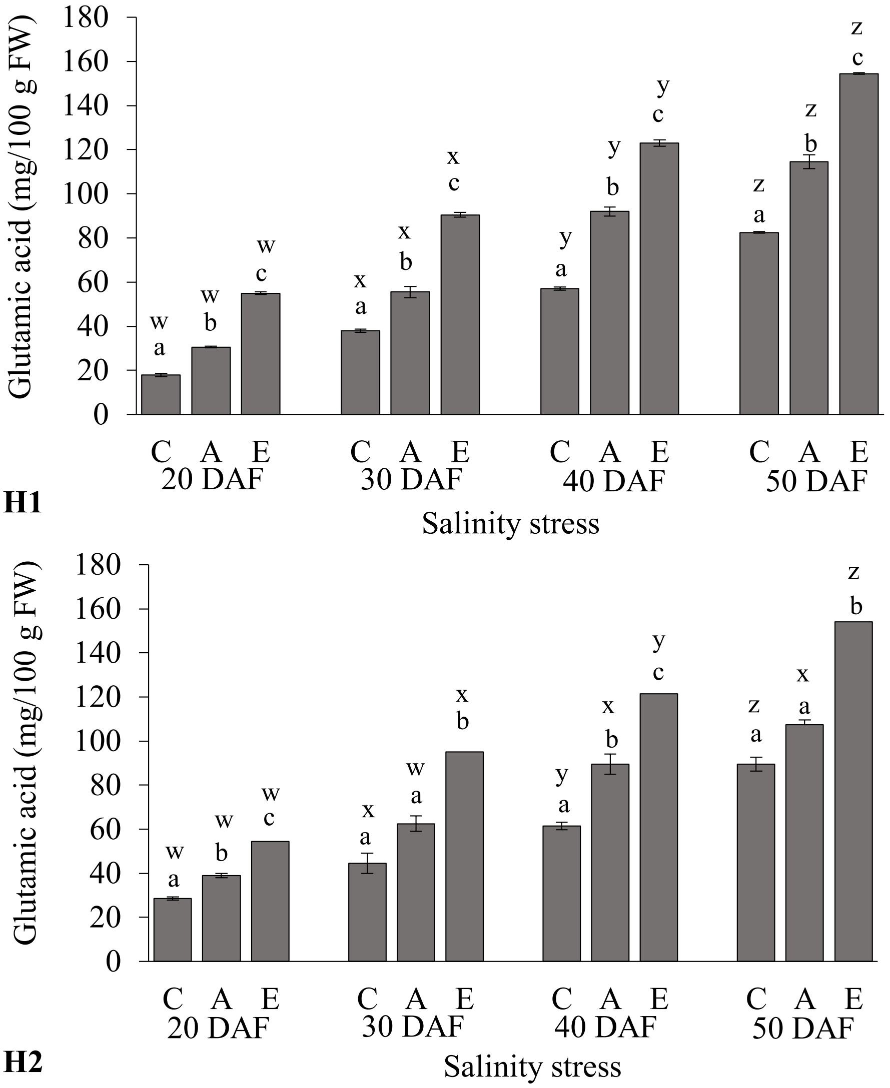


Fig. 6-H. Glutamic acid content (mg/100 g fresh weight [FW]) at four stages of fruit maturation in the chili pepper 'Habanero' (H). Graphs H1 show the results of the year 2018, and H2 show the results of the year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, *P* < 0.05). Error bars indicate the standard error.

Capsaicinoid content

Capsaicinoids were detected only in ‘Sapporo’ and ‘Habanero’ fruit. The patterns of the capsaicinoid content tended to be similar to those of the total sugar content, the Brix, and the glutamic acid content. The capsaicinoid content increased with increasing delay in harvesting of the fruit, and with increasing soil salinity. Among all of the cultivar, the E group of ‘Habanero’ harvested at 50 DAF in 2019 showed the highest capsaicinoid content, and the lowest capsaicinoid content was seen in the C group of ‘Sapporo’ harvested at 20 DAF in 2018.

‘Sapporo’ fruit of the E group had a significantly higher capsaicinoid content than the fruit of the C group at all DAF in 2018 (Fig. 7-S1) and 2019 (Fig. 7-S2). A significant difference in the capsaicinoid content of ‘Habanero’ fruit was observed between all groups at all DAF in 2018 and 2019, with the highest capsaicinoid content seen in the E group harvested at 50 DAF (Fig. 7-H1, 7-H2).

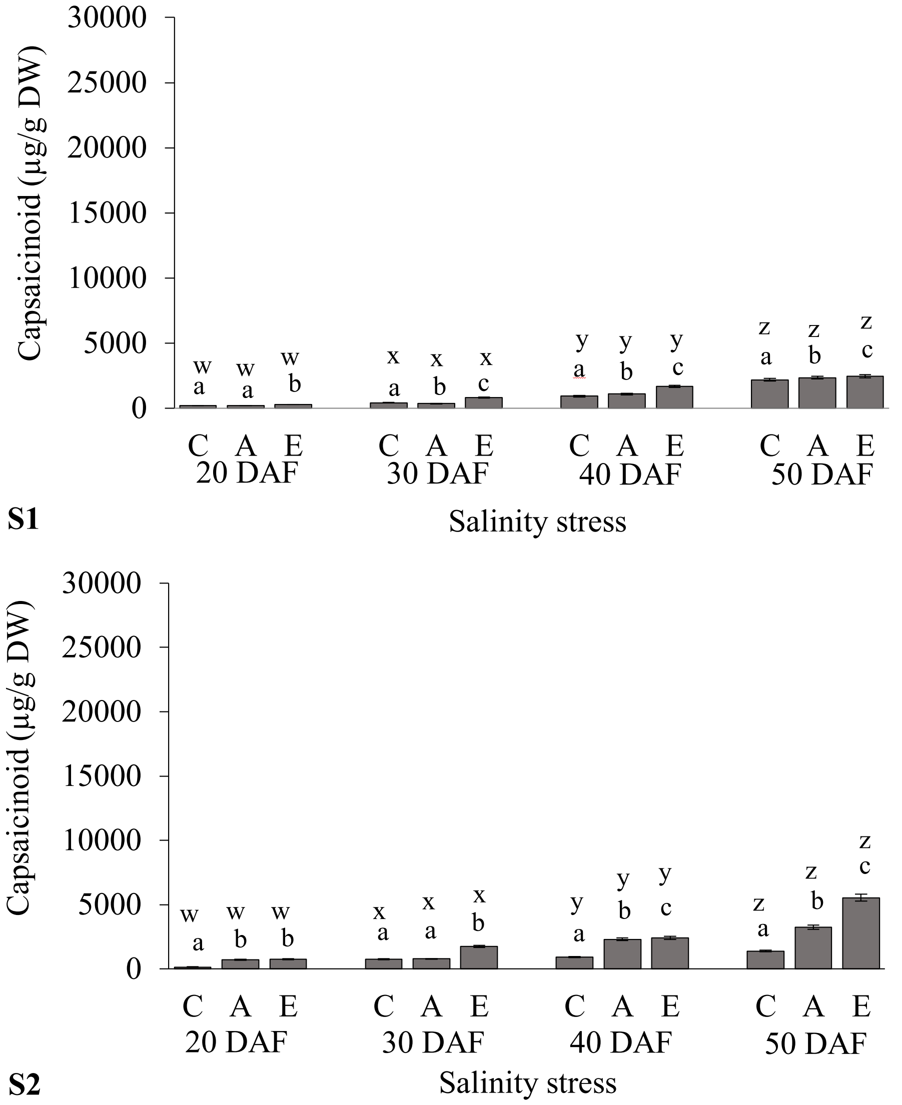


Fig. 7-S. Capsaicinoid content (µg/g dry weight [DW]) at four stages of fruit maturation in chili pepper 'Sapporo' (S). Graph S1 represent the year 2018 and S2 represent year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, $P < 0.05$). Error bars indicate the standard error.

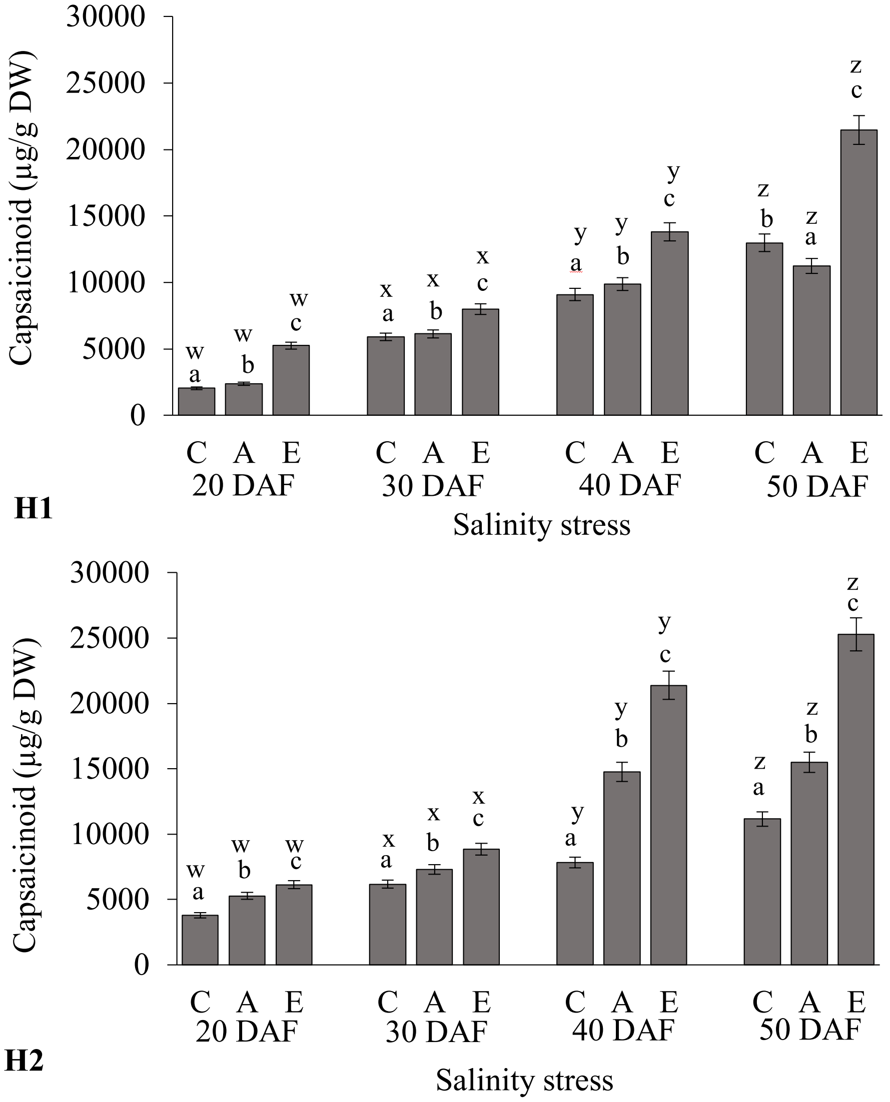


Fig. 7-H. Capsaicinoid content (µg/g dry weight [DW]) at four stages of fruit maturation in chili pepper 'Habanero' (H). Graph H1 represent the year 2018 and H2 represent year 2019. Fruit was sampled at 20, 30, 40, and 50 days after flowering (DAF). Different lowercase letters, a, b, and c, above the bars for the same DAF, and different lowercase letters, w, x, y, and z, for the same treatments (control, C; additional salinity stress, A; and excessive salinity stress, E) indicate significant differences between the treatment groups and between the DAF, respectively (Tukey's pairwise test, *P* < 0.05). Error bars indicate the standard error.

Discussion

Among all of the cultivar in the present study, the C group of ‘Fushimiamanaga’ harvested at 20 DAF in 2018 and 2019 showed the highest glucose content, followed by the C group of ‘Manganji’ in both years. Moreover, among all of the cultivar, the E group of ‘Manganji’ harvested at 50 DAF in 2018 and 2019 showed the highest total sugar content, followed by the E group of ‘Fushimiamanaga’ in both years. According to Hatakeyama et al. (2017), from among 39 strains tested, these two cultivar showed the highest glucose content and total sugar content. The highest Brix percentage was seen in the E group of ‘Fushimiamanaga’ harvested at 50 DAF in 2018; however, in 2019, the highest Brix percentage was seen in the E group of ‘Sapporo’ harvested at 50 DAF. Even in 2018, after ‘Fushimiamanaga’, the E group of ‘Sapporo’ showed the highest Brix percentage, and it was very close to that of “Fushimiamanaga’. These results are in agreement with those of Hatakeyama et al. (2017), who showed that ‘Fushimiamanaga’ and ‘Sapporo’ had the highest Brix percentage among the 39 strains of Chili pepper tested. Among all of the cultivar, the E group of ‘Manganji’ harvested at 50 DAF in 2019 and 2018 showed the highest glutamic acid content. This result was also in complete agreement with that of Hatakeyama et al. (2017), who showed that ‘Manganji’ had the highest glutamic acid content among the 39 strains of Chili pepper tested.

During the experimental period in 2018, some stressed plants exhibited leaf burn, and most of their leaves fell off. However, after stopped inducing the salinity stress, the damaged plants recovered, and then continued all treatments application. The leaf burning and loss of leaves may have been due to the combination of high-temperature stress and salinity stress during that period. It was reported that comparable salinity levels (2.5 and 4.1 dS/m) severely damaged ‘Habanero’ plants grown in the field (Niu et al., 2010), whereas all plants in this experiment were grown in a greenhouse, and all plants survived throughout this study. This contrast may be due to the different experimental conditions used, which enabled my damaged plants to recover within a short period of time in the present experiment. Also, plants grown in the field may be exposed to higher salinity levels than those applied in my experiment due to soil reactions and soil physical characteristics. Moreover, in the experiment conducted by Niu et al. (2010), the treatments were started from a younger stage, while present experiment I started at the onset of flowering.

According to Najet et al. (2016), no significant accumulation of soluble sugars was detected in salinity-stressed plants in a pot experiment; however, the largest accumulation of soluble sugars was found with an excessive salinity level (170 mM) for cultivar Skhira (*Capsicum annuum*). Also, in 'Caballero' Chili pepper (*C. frutescence*), a 20% increase in soluble sugars was observed in fruit grown at high salinity levels (Azuma et al., 2010). According to Saito et al. (2008), soil salinity can be responsible for changes in fruit metabolism and physiology. Comparing the present results with those of the above studies, the total sugar content and the Brix of the fruit tended to increase with increasing salinity stress. However, the fruit size decreased with increasing salinity stress.

Previous studies have shown that in the case of tomatoes, the sugar concentrations of fruit can be increased by applying water or salinity stress to plant root zones before harvesting (Adams and Ho, 1992; Ehret and Ho, 1986; Saito et al., 2006). Although increased sugar concentrations can be achieved by starting stress treatments after early fruit set, reductions in fruit weight due to the effects of strong stress are observed (Okano et al., 2002). On the other hand, increases in sugar and total soluble solids in tomato fruits subjected to salt or water stress result from decreased water uptake (Bolarin et al., 2001;

Chretien et al., 2000; Ehret and Ho, 1986; Li et al., 2001). However, because of the trade-off between an increased sugar concentration and a decreased fruit size, the final yields per plant are markedly reduced. Saito et al. (2006) and Sakamoto et al. (1999) have also reported that the addition of NaCl to nutrient solutions increases tomato fruit sugar concentrations, but it tends to decrease fruit yield. According to Nielsen et al. (1991) and Tadesse et al. (2002), fruit can be harvested in the green, transition between green and red, or red states, but the harvest time is crucial for fruit quality, since hexose sugars accumulate during ripening, this can be cause in major to increase the total sugar concentration in the fruits. The present study also showed that the delayed harvesting of Chili pepper appeared to increase the total sugar content of the fruit of Chili pepper.

Also, in the present study, the content of glucose showed a trend opposite to those of the total sugar content and the Brix. However, it has been reported that high salinity treatments (7 dS/m) caused a significant increase in fruit glucose levels in 'Habanero' Chili pepper (Rafael et al., 2014). It is possible that the experiment performed by Rafael et al. (2014) used a Hoagland-based nutrient solution, and they used $CaCl_2$ to prevent sodium-induced calcium deficiency; these can be responsible for changes in fruit metabolism, and can cause an increase in glucose content. However, the present experiment was carried out in soil-based medium in plastic pots with NaCl added via the water supply, and I did not apply $CaCl_2$ to the soil; these practices may be the cause of the opposing trends seen in the glucose content in the present experiment. Previous studies have examined the effect of salinity stress at different growth stages in a low-truss, high-density tomato production system (Saito et al., 2006; Sakamoto et al., 1999), but a successful method for increasing the sugar concentration by increasing soil salinity remains to be developed. Saito et al. (2006) reported that the Brix of tomato fruit increased from 6.1% to 9.9% in a third-truss, high-density tomato production system when nutrient solution EC was increased and maintained at 8.0 dS/m from first truss anthesis until fruit harvesting by adding NaCl. Wu and Kubota (2008) also reported that the Brix of tomato fruit increased from 5.3% to 6.1% when nutrient solution EC was increased to 4.5 dS/m at the pink stage. The current study results were also in agreement with the above results; I found that the Brix of Chili pepper fruit increased from 9% to 14.9% when the soil salinity stress condition was increased (10 dS/m).

The glutamic acid content also tended to increase with increasing soil salt content and with increasing delay of harvesting. According to Nonaka et al. (2012), the glutamic acid content of the red fruit (<60 DAF) of ‘Botankosho’ (*Capsicum annuum*) is three times higher than that of the green fruit (<50 DAF). My present results agree with those of Nonaka et al. (2012) since the red fruits in the present experiment (40 and 50 DAF) showed a significantly higher glutamic acid content than the immature harvested fruits. In addition, according to chapter II, drought stress and late harvesting cause a significant increase in the glutamic acid content of the fruits of ‘Manganji’, ‘Fushimiamanaga’, and ‘Sapporo’. Regarding those cultivar, the present results also showed that excessive salinity stress and late harvesting cause a significant increase in the glutamic acid content.

Pungency is related to environmental conditions, and it increases in response to stressful conditions (Harvell and Bosland, 1997). In habanero ‘Orange’, high salinity conditions (7 dS/m) marginally increased the pericarp capsaicin content. Similar trends in capsaicinoid concentrations were found in ‘Jalapeno’ Chili pepper (*Capsicum annuum*) under salt treatments (Arrowsmith et al., 2012). Similar results were shown in the present study: capsaicinoid content increased with fruit development and with increasing soil salinity. Moreover, Rafael et al. (2014) revealed that capsaicin and dihydrocapsaicin levels were highest with high salinity treatments (Jalapeno Chili pepper, 179.9 mg/100 g, at 7 dS/m salinity level).

When plants were grown under an excessive salinity stress condition, the size and weight of the produced fruit were decreased when compared to those grown under the control condition. This tendency was common in almost all varieties and treatment groups. According to Zhani et al. (2012), results on the fresh and dry weights of five cultivar of Tunisian Chili pepper (*C. frutescens*) showed that the fresh and dry biomasses of all cultivar decreased significantly with increasing NaCl concentration. Also, salinity stress had a significantly negative impact on the plant height of the five Chili pepper studied by Zhani et al. (2012). The present study also showed a similar tendency for plants grown under salinity stress.

The present study clarified the relationships among fruit growth, salinity stress, and the contents of taste components (sugars, glutamic acid, and capsaicinoids) in Chili pepper fruit. However, to better understand how environmental factors affect the changes in the contents of taste components, further investigations are necessary on the relationships among fruit yield, fruit size, fruit water content, leaf number, photosynthesis ability, and the contents of taste components. In addition, it is necessary to elucidate the genetic mechanism by conducting expression analyses of the genes involved in the synthesis and accumulation of taste components.

Chapter IV

Effect of Stress Condition to Pod Parameters, yield, and Plant Growth in Chili pepper

Abstract

The taste components are very important factors when chili pepper using as a vegetable. But in case of adverse environmental conditions are effects to the taste components of the chili pepper. In many plants, such stresses are known to have a negative effect on growth as well as altering the various component contents of the harvest. Therefore present experiment is conducted to the find out the effect of environmental stress (water and salinity) to the Brix, capsaicinoid content, pod parameters, yield and plant growth in chili pepper. Experiment was conducted at the green house condition in Research field of Shinshu University. Used 'Sapporo', 'Shishito', and 'Botankosho' (*Capsicum annuum*) varieties, and planted in plastic pots. To the experiment I, used three volumes of water stress in individual applications: drought (50 mL water), standard water supply (130 mL water), and excess water supply (260 mL water). To the experiment II, used three salinity levels applied using sodium chloride (NaCl); excessive salinity (10 dS/m), additional salinity (5 dS/m), and the normal salinity as control (0.9 dS/m). Within the cultivation period I collected Brix value, capsaicinoid content, number of fruits, fruit weight, total yield, number of leaves, number of branches, and plant height in per plant basis. I able to found that stress during cultivation increased Brix and capsaicinoid content, but also decreased yield, fruit size, and leaf number. The stress-induced reduction in Brix could be due to the reduction in yield and fruit size, but

similarly, the negative impact of reduced photosynthetic capacity due to reduced leaf number on Brix was inferred to be less.

Introduction

Chili peppers belong to the genus *Capsicum* in the Solanaceae family, and are an important source of spices because their capsaicinoids give a sensation of hotness when eaten. Chili peppers are popular as vegetables (fresh or cooked) as well as spices in most parts of the world, and thus it is important to maintain fruit quality to supply the best product. Many physiological factors affect the quality of the fruit, such as size, shape, and color. In addition, flavor, aroma, nutrient content, and taste components (sugar, glutamic acid, Brix, etc.) are also important factors. The perception of flavor is influenced by many factors, and taste (sweetness, sourness) is determined basically by sugars and acids (Kader et al., 1977).

Moreover, stress conditions such as water- and salinity-stress can increase the contents of taste components in chili pepper fruits (Rathnayaka et al., 2020, and Rathnayaka et al., 2021). The productivity of chili peppers is also often reduced by both biotic and abiotic stresses, the types of which can vary among regions (Barchenger and Bosland, 2019). Changes in climatic factors, such as temperature, precipitation, and the frequency and severity of extreme weather events, such as drought, floods and windstorms, directly affect crop yields. Plant growth, development, and yield of chili peppers is also influenced by climate change. It is thus also important to discover the effects of environmental stresses (water/salinity) on Brix, plant growth parameters, and yields in chili peppers. Andrew, (1995) indicated that there is a significant variance in stress tolerance among pepper plants, mainly depending on their origin. Also, Jamez et al. (1999) reported that fruit production under water deficit conditions was affected in different degrees depending on the cultivar. Soil acidity/salinity and drought are the most deleterious abiotic stresses affecting crop productivity and are responsible for significant crop loss globally. Acidity and salinity makes the soil unsuitable for crop cultivation due to various reasons like immobilization of nutrients like phosphorus and increased levels of toxic forms of elements such as aluminum and iron. According to Brunner and Sperisen (2013), about 40% of the global arable land is acidic and in south and south-east Asia, more than 100 million ha of land is unused due to soil acidity.

It is thus also important to discover the effects of environmental stresses (water/salinity) on Brix, plant growth parameters, and yields in chili peppers. The present study was conducted to clarify the how Brix, which changes in response to environmental stress, is related to other traits such as crop yield.

Materials and Methods

Plant materials and experimental design

The experiment was conducted from April to October 2020 in a greenhouse at the experimental farm (773 m a.s.l.) of AFC, Faculty of Agriculture, Shinshu University in Minamiminowa, Nagano, Japan. Water stress and salinity stress treatments and other practices were the same in chapter II and III. Therefore, as stress treatments, applied three volumes of water in individual applications to represent drought (D; 50 mL water per application), standard water supply (S; 130 mL water per application), and excess water supply (E; 260 mL water per application). And applied three salinity levels using sodium chloride (NaCl; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan): normal salinity control (C; 0.9 dS/m (0.57 g/L)), additional salinity (A; 5 dS/m (3.2 g/L)), and excessive salinity (E; 10 dS/m (6.4 g/L)).

For water stress treatments, we used three Japanese cultivars of chili pepper, ‘Shishito’ (seeds purchased from Takii Seed Co., Ltd. Kyoto, Japan); the local pungent variety 'Botankosho', which is used as a traditional vegetable in northern Nagano Prefecture (seeds donated by the Madarao Botankosho Conservation Society (Nakano, Nagano, Japan)); and ‘Sapporo Oonaga Nanban’ (‘Sapporo’; Tsurushin Seed, Matsumoto, Japan), which is a local variety originating in Hokkaido (Fig. 1). For salinity stress treatments, we used ‘Shishito’ and ‘Sapporo’ cultivars.

Before treatments, seedlings of approximately 150 mm in height were transplanted to plastic pots (diameter 18 cm, volume 1.8 L) filled with 1 kg of the same commercial potting medium as that used in experiment 2, and the stress treatments were applied starting 1 week after the seedlings were transplanted. All flowers including flower buds were removed before applying stress treatments. During the stress treatment, each flower was tagged at the end of anthesis to ensure that the fruit could be harvested at around 30 DAF. Fruits harvested for each treatment were used for measuring Brix and capsaicinoid contents in the placental septum at minimum and maximum stress conditions. In addition, data was collected as harvested number of fruits, fruit weight, total yield, number of leaves, number of branches, and plant height were measured on a per plant basis. A randomized complete block design was used for the analysis of experimental data.

During the cultivation period, a single application of slow-acting home gardening fertilizer (N:P:K, 10:10:10; Shizen Oyokagaku Co. Ltd., Nagoya, Aichi, Japan) was applied in August of each year. The water was applied with consideration of the daily temperature and weather. On sunny days and/or when the daily temperature exceeded 30°C, water was applied three times per day. On rainy or cloudy days and/or when the daily temperature was less than 30°C, water was applied twice per day. Throughout the experiment, other management practices were applied equally to all plants in the same greenhouse.

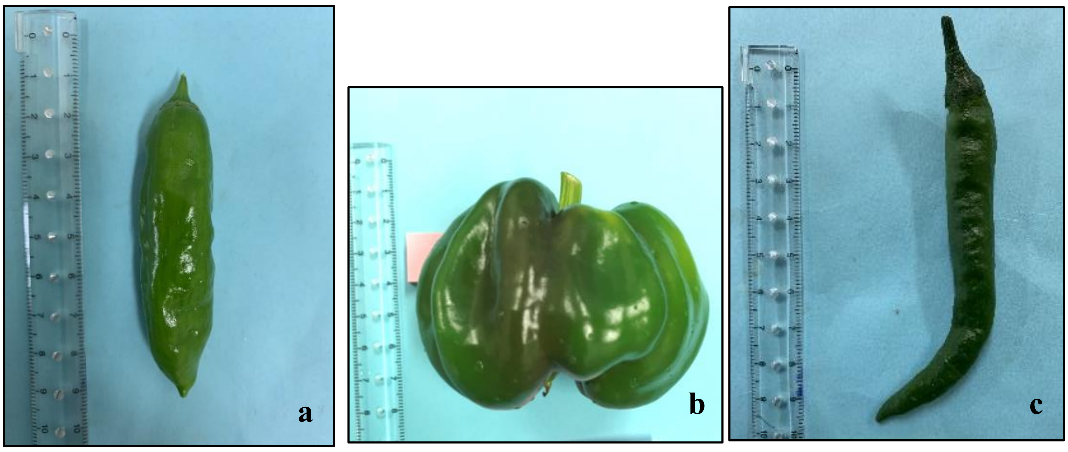


Fig. 1. Fruit of Japanese chili pepper (*Capsicum annuum*) cultivars included in the study: (a) 'Shishito' (Takii), (b) 'Botankosho' (Local), and (c) 'Sapporo Oonaga Nanban' (Tsurushin).

Solution preparation for analysis of Brix

Brix is primarily a measure of the sugar concentration in a solution. Extracts were prepared from fruit tissue ground using a grinder (YMB-400, Yamazen) followed by filtering through 125 mm filter paper (ADVANTEC$^{®}$). Extracts were used directly to measure the Brix value with a digital portable refractometer (Pen-J, Atago Co. Ltd, Tokyo, Japan).

Capsaicinoids analysis - HPLC apparatus and analysis conditions

The HPLC analysis conditions were as follows: LC column (50 × 3.0 mm; Shimadzu Corporation, Kyoto, Japan), column temperature 40°C, mobile phase 70% methanol, flow rate 1 mL/min, and absorbance at 280 nm. To validate the analytical conditions, capsaicin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a standard. Standard capsaicin solutions of 62.5, 125, 250, and 500 µg/mL (Othman et al., 2011) were analyzed, and a calibration curve was prepared. Sample solutions were prepared using freeze-dried chili pepper for 24h, and capsaicin was extracted after ground chili pepper powder (2 mg) was added to methanol (20 mL) and being kept for 1 h at 40°C.

Results

Plant height

Plant height of all cultivars tended to decrease with increasing drought or salinity stress. In ‘Shishito’ and ‘Sapporo’, a significant difference in the plant height was seen between excess water stress and drought stress conditions (Table 1). Significant differences in plant height were seen between all water stress conditions in ‘Botankosho’. The lowest plant heights were observed in drought stress conditions in all cultivars. Salinity stress-treated plants behaved the same as those under water stress (Table 2). In ‘Shishito’, significant differences in plant height were seen between all salinity stress treatments. A significant difference in plant height was seen between control and excess salinity stress treatment in ‘Sapporo’. Correlation between Brix and total sugar content was not found in either cultivar group ‘Sweet pepper/ Paprika’.

Number of branches

The number of branches of all cultivars tended to decrease with increasing drought or salinity stress. In ‘Shishito’, significant differences in the number of branches were seen between all water stress treatments (Table 1). A significant difference in the number of branches was seen between excess water stress and drought stress conditions in ‘Sapporo’ and ‘Botankosho’. The lowest numbers of branches were observed in drought stress conditions in all cultivars. Salinity stress-treated plants behaved the same as those under water stress (Table 2). A significant difference in the number of branches was seen between control and excess salinity stress conditions in ‘Shishito’ and ‘Sapporo’.

Number of leaves

The number of leaves of all cultivars tended to decrease with increasing drought or salinity stress. Significant differences in the number of leaves were seen between all water stress treatments in ‘Shishito’ and ‘Botankosho’. In ‘Sapporo’, a significant difference in the number of leaves was seen between excess water stress and drought stress treatments (Table 1). The lowest numbers of leaves were observed in drought stress conditions in all cultivars. Salinity stress-treated plants behaved the same as those under water stress (Table 2). Significant differences in the number of leaves were seen between all salinity stress treatments in ‘Shishito’. In ‘Sapporo’, a significant difference in the number of leaves was seen between control and excess salinity stress treatment.

Number of fruits

The number of fruits in all cultivars tended to decrease with increasing drought or salinity stress. Significant differences in the number of fruits were seen between all water stress treatments for all cultivars (Table 1). The lowest number of fruits was observed under drought stress conditions in all cultivars. Salinity stress-treated plants behaved the same as those under water stress (Table 2). Significant differences in the number of fruits were seen between all salinity stress treatments in ‘Shishito’ and ‘Sapporo’.

Fruit weight

The fruit weights of all cultivars tended to decrease with increasing drought or salinity stress. A significant difference in fruit weight was seen between excess water stress and drought stress conditions in ‘Shishito’ and ‘Sapporo’. In ‘Botankosho’, significant differences in fruit weight were seen between all water stress treatments (Table 1). The lowest fruit weights were observed in the drought stress condition for in all cultivars. Salinity stress-treated plants behaved the same as the water stress-treated plants (Table 2). Significant differences in fruit weight were seen between all salinity stress treatments in ‘Shishito’ and ‘Sapporo’.

Total yield

The total yield of all cultivars tended to decrease with increasing stress drought or salinity stress. Significant differences in the total yield were seen between all water stress treatments in ‘Shishito’ and ‘Botankosho’. In ‘Sapporo’, a significant difference in total yield was seen between excess water stress and drought stress conditions (Table 1). The lowest total yields were observed in drought stress conditions in all cultivars. Salinity stress-treated plants behaved the same as those under water stress (Table 2). A significant difference in the total yield was seen between all salinity stress treatments in ‘Shishito’ and ‘Sapporo’.

Brix

The Brix in the fruits of all cultivars tended to increase with increasing drought or salinity stress in the soil. Significant differences in Brix were seen between all water stress treatments and all cultivars in water stress treatment experiment (Table 1). The highest Brix was observed in the drought stress treatment in all cultivars. Salinity stress-treated plants behaved the same as the water stress-treated plants (Table 2). Significant differences in Brix were seen between all salinity stress treatments in ‘Shishito’, and a significant difference was observed between excess salinity stress treatment and control treatment in ‘Sapporo’.

Table 1. Effect of water stress for plant growth parameters, pod parameters, yield per plant and Brix % in ‘Shishito’ ‘Sapporo Oonaga Nanban’ and ‘Botankosho’ in 2020.

Varieties	Water stress	Plant Height (cm)	Nu. of Branches	Nu. of Leaves	Nu. of Fruits	Fruit Weight (g)	Total Yield (g)	Brix %
Shishito	E	78.3 ± 1.2 a	13.5 ± 0.4 a	249 ± 7.0 a	75.0 ± 0.3 a	10.5 ± 0.1 a	788 ± 33.1 a	5.6 ± 0.06 a
	S	68.0 ± 1.4 a	9.5 ± 0.2 b	161 ± 4.0 b	57.5 ± 0.6 b	9.4 ± 0.2 a	518 ± 21.9 b	7.5 ± 0.10 b
	D	44.8 ± 2.2 b	6.8 ± 0.3 c	87 ± 2.0 c	21.3 ± 0.7 c	5.4 ± 0.1 b	106 ± 8.8 c	10.4 ± 0.05 c
Sapporo	E	84.5 ± 2.7 a	11.8 ± 0.5 a	214 ± 9.6 a	59.8 ± 2.6 a	8.6 ± 0.1 a	532 ± 23.1 a	6.8 ± 0.55 a
	S	49.0 ± 1.6 b	8.0 ± 0.2 b	167 ± 7.9 ab	33.5 ± 1.6 b	8.5 ± 0.1 a	291 ± 14.3 b	8.0 ± 0.06 b
	D	44.3 ± 1.5 b	7.0 ± 0.2 b	115 ± 2.8 b	23.8 ± 0.7 b	7.1 ± 0.1 b	169 ± 4.7 b	10.7 ± 0.02 c
Botankosho	E	85.0 ± 1.7 a	11.8 ± 0.3 a	189 ± 9.0 a	29.3 ± 1.4 a	51.0 ± 0.4 a	1404 ± 19.4 a	5.6 ± 0.06 a
	S	62.3 ± 2.0 b	9.3 ± 0.3 a	132 ± 2.2 b	24.8 ± 1.0 b	35.8 ± 0.4 b	866 ± 8.7 b	7.2 ± 0.17 b
	D	47.8 ± 1.4 c	47.8 ± 0.2 b	78 ± 4.3 c	13.8 ± 1.9 c	28.5 ± 0.8 c	385 ± 7.0 c	8.5 ± 0.12 c

E, S and D denotes excess water supply, standard water supply and drought stress, respectively. Treatments with the same letter do not differ significantly within a variety by Tukey’s pairwise test ($P < 0.05$). Values are means ± SE. n = 3

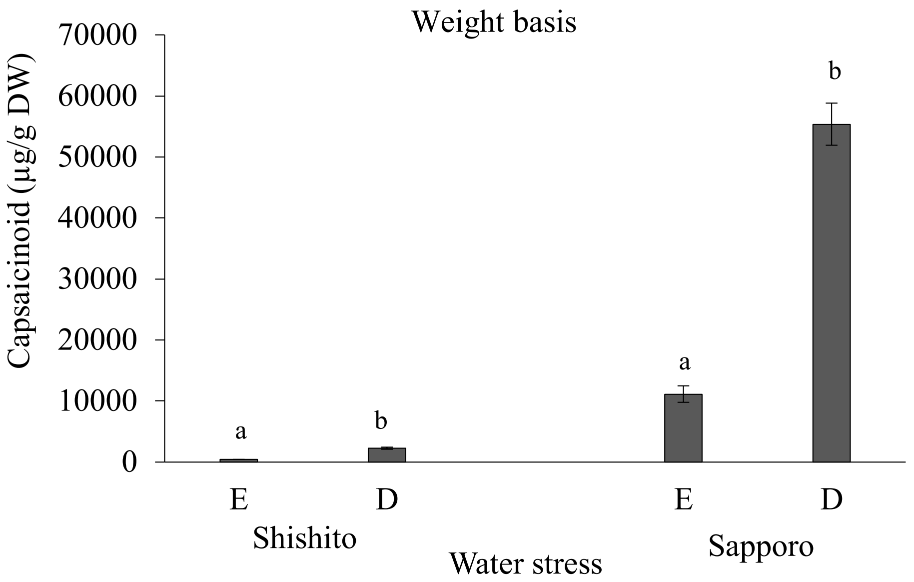
Table 2. Effect of salinity stress for plant growth parameters, pod parameters, yield per plant and Brix % in ‘Shishito’ and ‘Sapporo Oonaga Nanban’ in 2020.

Varieties	Salinity Stress	Plant Height (cm)	Nu. of Branches	Nu. of Leaves	Nu. of Fruits	Fruit weight (g)	Total Yield (g)	Brix %
Shishito	E	55 ± 1.2 a	5.5 ± 0.1 a	69.5 ± 6.4 a	43 ± 1.1 a	5.2 ± 0.06 a	213 ± 5.3 a	9.9 ± 0.06 a
	A	67 ± 1.0 b	9.0 ± 0.2 b	131.3 ± 4.8 b	53 ± 0.9 b	7.3 ± 0.10 b	369 ± 6.3 b	8.6 ± 0.05 b
	C	81 ± 1.5 c	10.5 ± 0.3 b	209.3 ± 3.0 c	73 ± 1.3 c	9.0 ± 0.09 c	657 ± 11.5 c	7.9 ± 0.01 c
Sapporo	E	34 ± 0.7 a	7.3 ± 0.2 a	88.8 ± 2.0 a	24 ± 2.2 a	6.2 ± 0.06 a	150 ± 13.4 a	10.4 ± 0.12 a
	A	44 ± 2.1 a	8.0 ± 0.2 a	127.0 ± 2.2 a	52 ± 1.5 b	7.2 ± 0.05 b	371 ± 10.4 b	9.6 ± 0.06 b
	C	62 ± 2.9 b	11.0 ± 0.2 b	183.8 ± 12.6 b	70 ± 2.9 c	9.2 ± 0.07 c	638 ± 26.4 c	8.7 ± 0.15 c

E, A and C denotes excess salinity stress, additional salinity stress and control, respectively. Treatments with the same letter do not differ significantly within a variety by Tukey’s pairwise test (*P* < 0.05). Values are means ± SE. n = 3

Capsaicinoids in placental septum

Capsaicinoid contents in placental septum significantly increased under drought stress conditions and excess salinity stress condition in 'Shishito' and 'Sapporo' (Fig. 2 and 3).



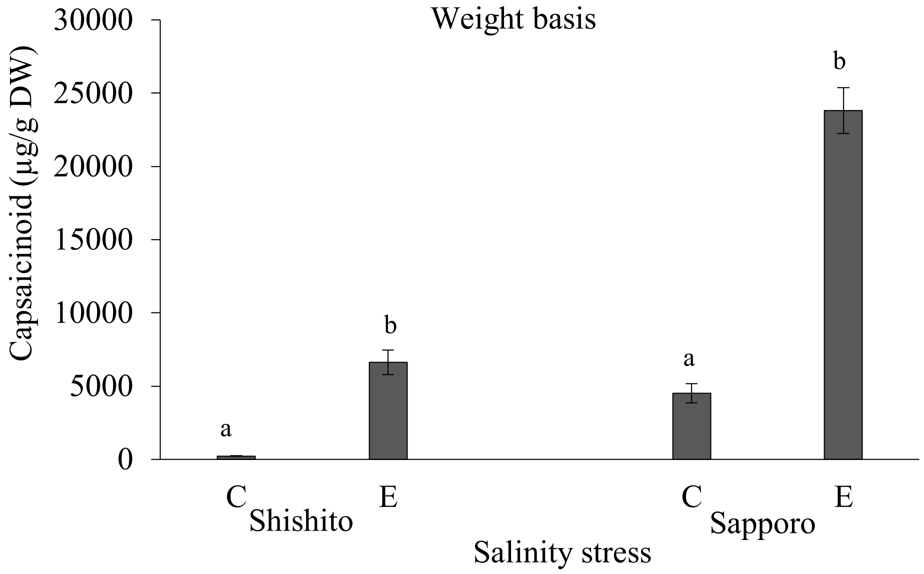


Fig. 2. Capsaicinoids in placental septum of chili peppers on the dry weight basis of fruit in ‘Shishito’ and ‘Sapporo Oonaga Nanban’ for both stress conditions in 2020. In water stress; E and D denotes excess water supply and drought stress, respectively. In salinity stress; C and E denotes control and excessive salinity stress, respectively. Treatments with a different letter for the same variety differ significantly by Tukey’s pairwise test (*P* < 0.05).

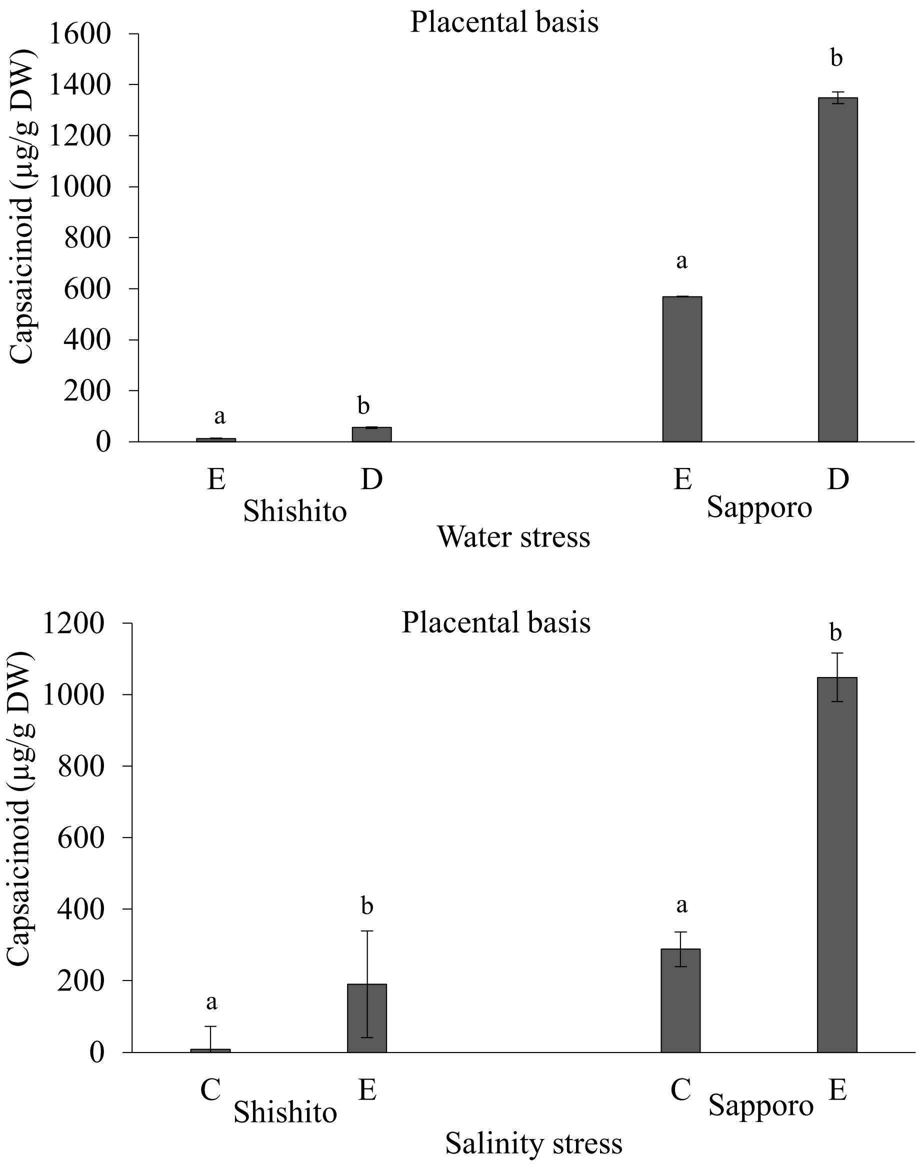


Fig. 3. Capsaicinoids in placental septum of chili peppers on the placental septum basis in ‘Shishito’ and ‘Sapporo Oonaga Nanban’ for both stress conditions in 2020. In water stress; E and D denotes excess water supply and drought stress, respectively. In salinity stress; C and E denotes control and excessive salinity stress, respectively. Treatments with a different letter for the same variety differ significantly by Tukey’s pairwise test (*P* < 0.05).

Discussion

When plants were grown under drought or excessive salinity stress conditions, Brix became larger than when grown under excess water or normal conditions. This tendency was common in almost all varieties and treatments in both experiments. Rathnayaka et al. (2021) found that Brix significantly increased in chili pepper fruits when plants are under drought stress. The present results agreed with this for all cultivars under drought stress. Rathnayaka et al. (2021). Also found that Brix significantly increased in chili pepper fruits when plants are under salinity stress condition. The results in chapter IV obtained where excess salinity stress conditions were given also agreed with those of Rathnayaka et al. (2020).

Similarly, stress also reduced fruit yield and fruit size. Therefore, the increase of Brix in the fruits was inferred to be due to the concentration of sugar resulting from reduced number and size of fruits. However, stress also reduced the number of leaves, which should have limited the supply of sugar. The present results suggest that the sugar content in fruits increased because the sugar accumulation due to the decrease in fruit number and size was greater than the decrease in photosynthetic capacity caused by the stress-induced decrease in leaf number.

Capsaicinoid content showed a similar response to stress conditions and Brix. The highest content of capsaicinoids in placental septum was observed in the drought and excess salinity stress treatments. According to Estrada et al. (1999), stronger pungent chili fruits are found when chili peppers are grown under drought stress. Moreover, Rafael et al. (2014) revealed that capsaicin and dihydrocapsaicin levels were the highest in high salinity treatments. I found that the capsaicinoid content per weight (µg/g DW) of the placental septum was significantly increased by stress.

The size of fruits in drought and excess salinity stress conditions was small, while that in excess water and control salinity conditions was comparatively larger. If the placental septum size was not changed by the stress conditions even though the fruit size was reduced, and if the capsaicinoid synthesis capacity per fruit/placental septum will not change, then it may be that capsaicinoids was concentrated because of the small fruit size. Therefore, we analyzed the data in regard to stress conditions and capsaicinoids in the placental septum not on a weight basis but on a per placental septum basis (µg/fruit). However, in the present experiment, we found that the placental septum size was also reduced by stress condition (Fig. 4). According to these two analyses, it seems that the higher amount of capsaicinoids in stress conditions was not due to the concentration of them in a smaller fruit area (Fig. 3) but to accelerated capsaicinoid synthesis caused by stress conditions.

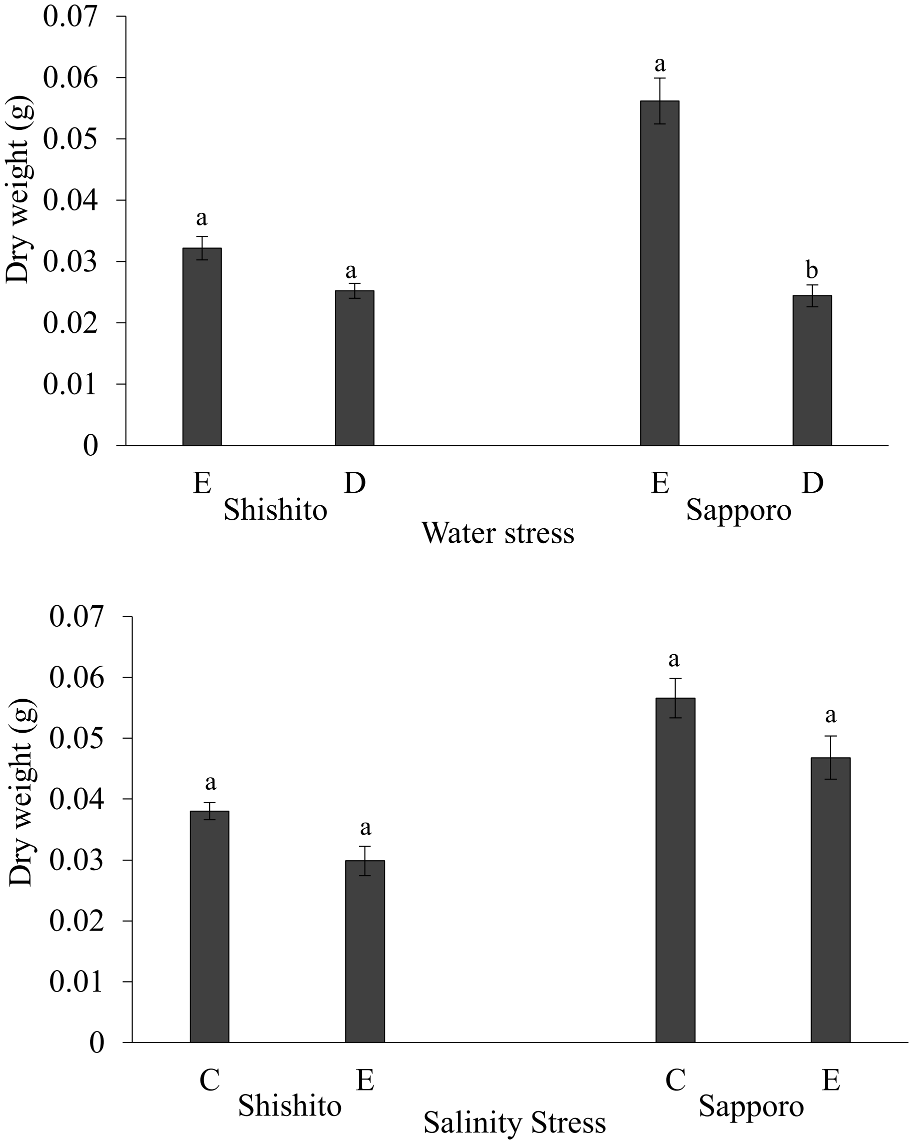


Fig. 4. Dry weights of placental septum in chili peppers ‘Shishito’ and ‘Sapporo Oonaga Nanban’ in 2020. In water stress; E and D denotes excess water supply and drought stress, respectively. In salinity stress; C and E denotes control and excessive salinity stress, respectively. Treatments with the same letter do not differ significantly within a variety by Tukey’s pairwise test ($P < 0.05$).

Plant growth parameters and fruit parameters including total yield was significantly decreased with the both stress condition conditions in the present experiment. It was mentioned buy previous researches, high soil salinity leads to poor germination, delays stand establishment, and reduces subsequent growth and yield (Niu and Rodriguez, 2010; Butt et al., 2016). Present experiment findings for salinity stress treatment also agreed with the growth and yield parameters with above findings. Moreover, occurrence of drought increases the concentration of salts in the soil which affects the reverse osmosis of loss of water from plant cells. This leads to poor plant growth and reduces productivity due to inhibition of several physiological and biochemical processes such as photosynthesis and respiration, etc. (Pena and Hughes, 2007).

The present study was conducted to clarify the relationship between fruit taste components, fruit parameters and plant growth parameters changes in response to environmental stress, which is related to other traits such as yield. I found that stress during cultivation increased Brix and capsaicinoid content, but also decreased yield, fruit size, and number of leaves. The stress-induced reduction in Brix could be due to the reduction in yield and fruit size, but similarly, the negative impact of reduced photosynthetic capacity due to reduced number of leaves on Brix was inferred to be less. However, the stress-induced increase in capsaicinoids content was not due to a decrease in fruit size, but rather due to an enhanced ability to synthesize capsaicinoids in the placental septum.

In the future, it will be necessary to elucidate the genetic mechanisms by conducting the expression analyses of the genes involved in the synthesis and accumulation of sugars and capsaicinoids when plants are under stress conditions.

Chapter V

Drought Stress Induced an Increase in the Pungency and Expression of Capsaicinoid Biosynthesis Genes in Chili Pepper (*Capsicum annuum* L.)

Abstract

The amount of taste components, including those for pungency, in chili pepper fruit (*Capsicum* spp.) is regulated by both environmental and genetic factors, and it changes depending on environmental factors, such as drought, salinity, and high temperatures. My previous study revealed that the amount of taste components, including capsaicinoid, was significantly increased in chili pepper fruits that were cultivated under a drought stress condition, and I assumed that such pungency fluctuations are regulated by the expression of genes involved in capsaicinoid synthesis. Therefore, the present experiment was conducted to determine the effect of drought stress on the pungency levels and expression levels of capsaicinoid biosynthesis genes in chili peppers. In the present study, I used Japanese chili pepper cultivars 'Shishito' and 'Sapporo Oonaga Nanban' (*C. annuum*), and cultivation was conducted in a greenhouse under a drought stress condition or an excess water supply condition. I harvested the fruits at 20 and 30 days after flowering (DAF). The fruits were used for morphological analysis, and the quantification of the capsaicinoid content in the placental septum was done using high performance liquid chromatography. Moreover, a gene expression analysis was carried out using quantitative reverse transcription polymerase chain reaction for 18 capsaicinoid biosynthesis genes. The results showed that 8 capsaicinoid biosynthesis genes (*ACL*, *pAMT*, *Pun 1*, *WRKY9*, *CaKR1*, *CaMYB31*, *KAS I*, and *FAT*) were significantly more highly expressed in the drought stress condition at 20 DAF in both cultivars. In addition, the *KAS III*, *BCKDH,*

and *BCAT* genes were also significantly more highly expressed in the drought stress condition at 20 DAF in ‘Shishito’. The *ACS* and *4CL* genes were also significantly more highly expressed in the drought stress condition at 20 DAF in ‘Sapporo’, and also at 30 DAF for *4CL*. However, the remaining genes, which were mostly involved in the phenylpropanoid pathway, did not show any significant differences in gene expression between the two conditions in either cultivar at all DAF.

Introduction

Capsicum annuum is a species of chili pepper that belongs to the Solanaceae family. It is widely grown for its fruit, and is consumed as a vegetable or as a spice after drying. The pungency of chili peppers is derived from a group of heat-producing alkaloids, including capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) and several related compounds, which are collectively termed capsaicinoids (Suzuki and Iwai, 1984). The greater the concentration of capsaicin, the greater the pungency of the fruit. Capsaicinoid synthesis occurs mainly in the placenta and interlobular septum of the fruit, and the capsaicinoid is accumulated in glandular vesicles located on the surface of the placental tissue (Iwai et al., 1979). The biosynthesis of capsaicinoids is accomplished by the condensation of vanillylamine and branched-chain fatty acid moieties, and these two precursors are derived from two separate pathways that constitute the capsaicinoid biosynthesis pathway (Arce-Rodríguez and Ochoa-Alejo, 2019), i.e., the phenylpropanoid pathway and the branched-chain fatty acid pathway. Many genes are known to be involved in the capsaicinoid biosynthesis pathway (Qin et al., 2014). Not only the genotype, but also the cultivation environment is known to affect the pungency level. As such, the pungency level may vary even among chili pepper fruits of the same variety that have the same genotype as a result of different cultivation conditions (Harvell and Bosland, 1997; Zewdie and Bosland, 2000). Also, capsaicinoid accumulation is associated with the developmental stage of the fruit (Estrada et al., 1997; Zewdie and Bosland, 2000). Furthermore, environmental factors, such as soil salinity stress, temperature, elevation, light exposure, and soil nutrients (nitrogen, phosphorus, and potassium) are known to cause changes in the pungency levels of the fruit of chili peppers. In water-stressed plants of *Capsicum chinense* Jacq., the capsaicin concentration increases in some parts of the fruit (Ruiz-Lau et al., 2011). My previous investigations in chapter II, found that taste components, including the capsaicinoid content, were significantly increased in chili pepper fruits that were cultivated under a drought stress condition. I considered that the expression of capsaicinoid biosynthesis genes is related to the drought stress-dependent fluctuation in pungency, and were interested in elucidating the genetic mechanism by conducting a gene expression analysis. Also, there have been few investigations regarding the influence of environmental factors, especially the water supply, on the pungency and expression of capsaicinoid biosynthesis genes in chili pepper fruit. Therefore, the present study was conducted to determine the relationship between drought stress, the content of capsaicinoids, and the expression of capsaicinoid biosynthesis genes.

Materials and Methods

Plant materials and experimental design

The experiment was conducted from April to October 2020 in a greenhouse at the experimental farm (733 m a.s.l) of the Education and Research Center of Alpine Field Science, Faculty of Agriculture, Shinshu University in Minamiminowa, Nagano, Japan. I used the local cultivars ‘Shishito’ (Takii and Co., Ltd., Kyoto, Japan), very low pungent chili cultivar in Japan and ‘Sapporo Oonaga Nanban’ (Tsurushin Seed, Matsumoto, Japan; hereafter ‘Sapporo’); ‘Sapporo’ is a Japanese pungent variety of the pepper. Seeds were sown on March 25, 2020, and subsequently, the seedlings were transplanted to plastic pots with a 75 mm diameter and filled with a commercial potting medium (Nae-ichiban; Sumitomo Forestry Landscaping Co., Ltd., Tokyo, Japan). During the seedling rearing period, the greenhouse was heated using oil heaters at night until early April to keep the temperature above a 15°C. On May 27, 2020, seedlings of approximately 150 mm in height were transplanted to clay pots (diameter of 30 cm) filled with the same commercial potting medium. During the cultivation period, slow-acting gardening fertilizer (N:P:K, 10:10:10; Shizen Oyokagaku Co., Ltd, Nagoya, Japan) was applied. The stress treatments were applied 1 week after the seedlings were transplanted. All flowers that were present were removed before the stress treatments were applied.

Two kinds of stress treatments were used: drought stress treatment and excess water supply treatment. For the drought stress treatment, 150 mL of water was applied for each irrigation, and for the excess water supply treatment, 780 mL of water was applied for each irrigation based on the pot size, as described in chapter II. In the excess water supply treatment, the excess water that overflowed from the pots was retained in a dish placed under the pot to allow absorption through the pot base. The number of times water was supplied was determined depending on the daily temperature and weather. When the temperature during the day exceeded 30°C or it was sunny, water was applied three times per day. When the temperature during the day remained below 30°C or it was rainy or cloudy, water was applied twice per day. During the stress treatment, each flower was tagged 1 day before flowering, and the fruits were harvested at 20 and 30 days after flowering (DAF). The placental septum weight, capsaicinoid content of the placental septum, and the expression levels of capsaicinoid biosynthesis genes were analyzed. Five individual plants of each cultivar were used for each treatment.

Pre-sample preparation for capsaicinoids analysis and RNA isolation

In the present study, capsaicinoid analysis and analysis of the expression levels of genes involved in the capsaicinoid biosynthesis pathway (Fig. 1) were carried out using separated placental septum tissues. The placental septum of fruit at 20 and 30 DAF were vertically separated; one half was lyophilized for capsaicinoid extraction, and the other half was kept at -80°C for RNA isolation.

Capsaicinoids analysis - HPLC apparatus and analysis conditions

As mentioned above, half of the placental septum was lyophilized using a freeze-dryer (FDU-200; Tokyo Rikakikai Co., Ltd., Tokyo, Japan). After determining the dry weight (DW) of samples, they were placed (0.09 to 0.25 g) into collection tubes with stainless-steel bases, and crushed using a Micro Smash TM MS-100 (Tomy Seiko Co., Ltd., Tokyo, Japan). Then, capsaicinoids (capsaicin and dihydrocapsaicin) were extracted in 5 mL of acetone. After transferring the supernatant to an eggplant-shaped flask, 2 mL of ethylacetate was added to the residue, and the supernatant was collected. The mixed supernatant was completely evaporated at 40°C using a rotary evaporator (N-1100; Tokyo Rikakikai). Finally, the extracted capsaicinoids were dissolved in 5 mL of methanol, and

10 μL of the extracted liquid was filtered and chromatographed using HPLC (LCsolution; Shimadzu Corporation, Kyoto, Japan).

In the HPLC analysis, separation was performed using a YMC-Pack ODS-A column (5 μm; 75 × 4.6 mm I. D.) coupled to a guard column (YMC-Guardpack ODS-A). The eluent consisted of methanol and distilled water (50:50), and the flow rate and temperature were set to 1.0 mL/min and 40°C, respectively. For detection with an ultraviolet detector, the wavelength was set to 280 nm. The capsaicinoid concentration (μg/g DW) was calculated based on the peak area of capsaicin and dihydrocapsaicin.

RNA isolation and quantitative reverse transcription polymerase chain reaction (RT-qPCR) of capsaicinoid biosynthesis genes

Total RNA was isolated from the other half of the placental septum with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). After removing the genomic DNA, the total RNA was used for RT-PCR, and cDNA was synthesized using a High-Capacity RNA-to-cDNA kit (ReverTra Ace® qPCR RT Master Mix with gDNA Remover, Toyobo Co., Ltd., Osaka, Japan). Then, qPCR was performed using the Step One Real-Time PCR System (Applied Biosystems). In the analysis, 18 capsaicinoid biosynthesis genes were analyzed as target genes, and actin was used as the reference gene. For gene amplification, I used the primers described by Tanaka et al. (2017), Koeda et al. (2019), and Han et al. (2019); the complete primer sequences are listed in Table 1. For PCR, PowerUp™ SYBR Green Master Mix (Applied Biosystems) was used for the PCR mixture, and the thermal cycle conditions described by Tanaka et al. (2017) were used, i.e., 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. Finally, the relative expression level was calculated based on the comparative CT method.

Table 1. Primers used for RT-qPCR.

Gene name		Primer sequence (5′-3′) Reference	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	
CaMYB31	F:	GACGAAAATGGAATGAAGAAGG	Han et al. (2018)
	R:	AGCATGGAGGTTCAAGATGATT	
Actin	F:	AGCAACTGGGACGATATGGAGAAG	Tanaka et al. (2017)
	R:	AAGAGACAACACCGCCTGAATAGC	

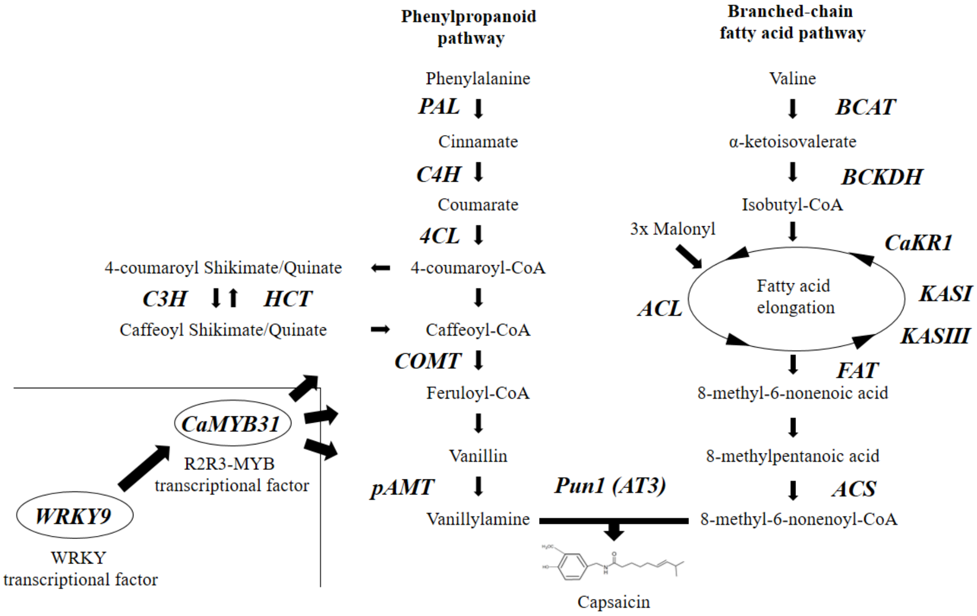


Fig. 1. Capsaicinoid biosynthesis pathway and the locations where the capsaicinoid biosynthesis genes analyzed in the present study are involved, except for *CaMYB31* and *WRKY9*. *PAL*: *phenylalanine ammonia lyase*, *C4H*: *cinnamate 4-hydroxylase*, *4CL*: *4-coumaroyl-coa ligase*, *HCT*: *hydroxycinnamoyl 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*. (Modified from Arce-Rodríguez and Ochoa-Alejo, 2019, and Koeda et al., 2019).

Results

Number of seed and Placental septum weight

In the drought stress condition, harvested fruits had a significantly lower number of seeds at 20 and 30 DAF in both cultivars with comparing excess water stress condition (Fig. 2). The fruits were also smaller and had a stumpy shape. The DW (g) of the placental septum was significantly smaller in the drought stress condition than in the excess water condition when harvested at 20 and 30 DAF in both cultivars except 30 DAF in 'Shishito' (Fig. 3).

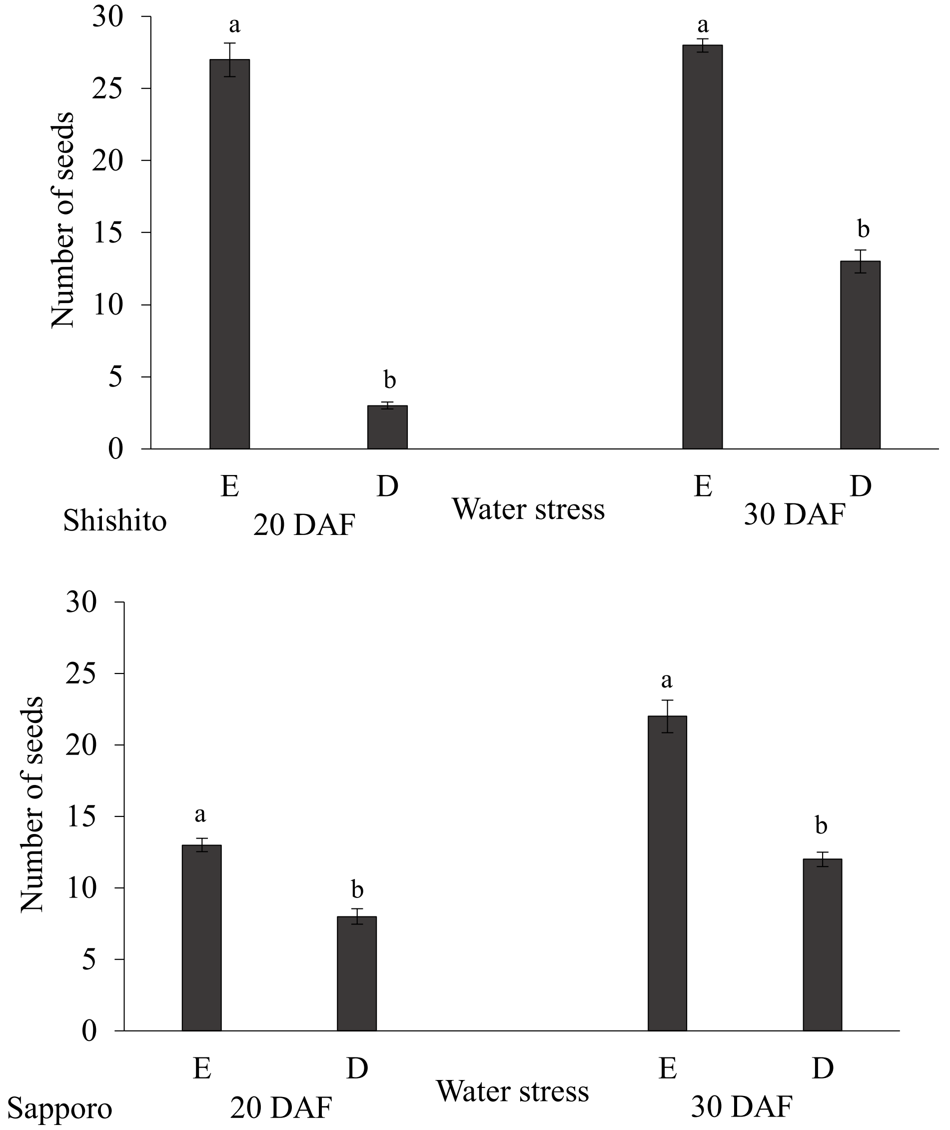


Fig. 2. Number of seeds at two stages of fruit maturation in chili peppers ‘Shishito’ and ‘Sapporo’ in 2020. Fruits were sampled at 20 and 30 days after flowering (DAF). Different lowercase letters (a, b) for the same DAF indicate significant differences between treatments (Tukey’s pairwise test, $P < 0.05$). D: drought, E: excess water supply. Error bars indicate the standard error.

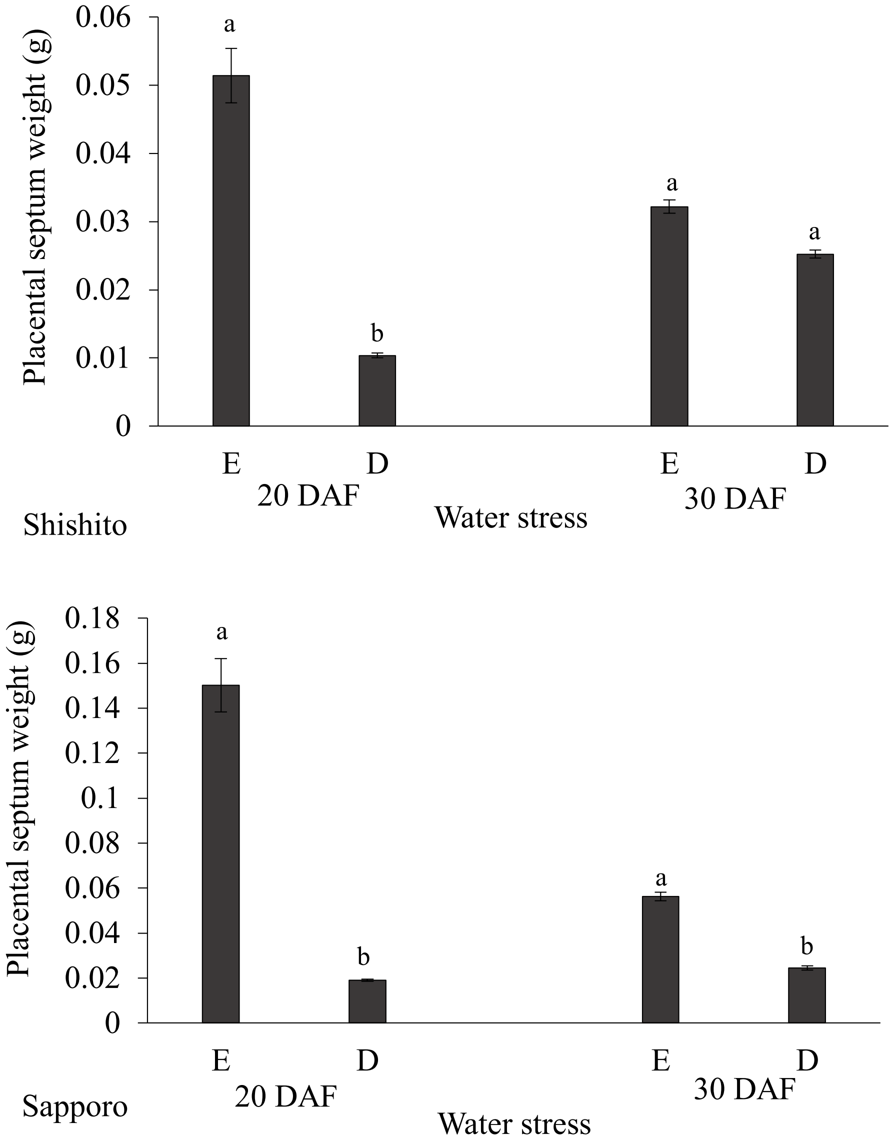


Fig. 3. Placental septum dry weight (g) at two stages of fruit maturation in chili peppers ‘Shishito’ and ‘Sapporo’ in 2020. Fruits were sampled at 20 and 30 days after flowering (DAF). Different lowercase letters (a, b) for the same DAF indicate significant differences between treatments (Tukey’s pairwise test, $P < 0.05$). D: drought, E: excess water supply. Error bars indicate the standard error.

Capsaicinoid content

I investigated the capsaicinoid concentration (capsaicinoid content per unit DW of placental septum) in the fruit harvested at 20 and 30 DAF, and compared the concentration among the two treatments. The capsaicinoid content was significantly higher in the drought stress condition than in the excess water supply condition in fruit harvested at 20 DAF (in ‘Shishito’, drought stress: 2252 $\mu g.g^{-1}$ DW, and excess water: 387 $\mu g.g^{-1}$ DW; in ‘Sapporo’, drought stress: 32499 $\mu g.g^{-1}$ DW, and excess water: 2766 $\mu g.g^{-1}$ DW). These results indicated that the capsaicinoid concentration increased under the drought stress condition (Fig. 4).

Additionally, I also compared the capsaicinoid content per placental septum, because the capsaicinoid concentration (capsaicinoid content per unit DW of placental septum) may vary greatly depending on the DW of the placental septum. Similar to the results of the capsaicinoid concentration, the capsaicinoid content of drought-stressed fruit tended to be significantly higher, and tended to increase with increasing harvesting DAF. These results indicated that drought-stressed fruit is more likely to be pungent than fruit grown under the other conditions regardless of the size of the placental septum (Fig. 5).

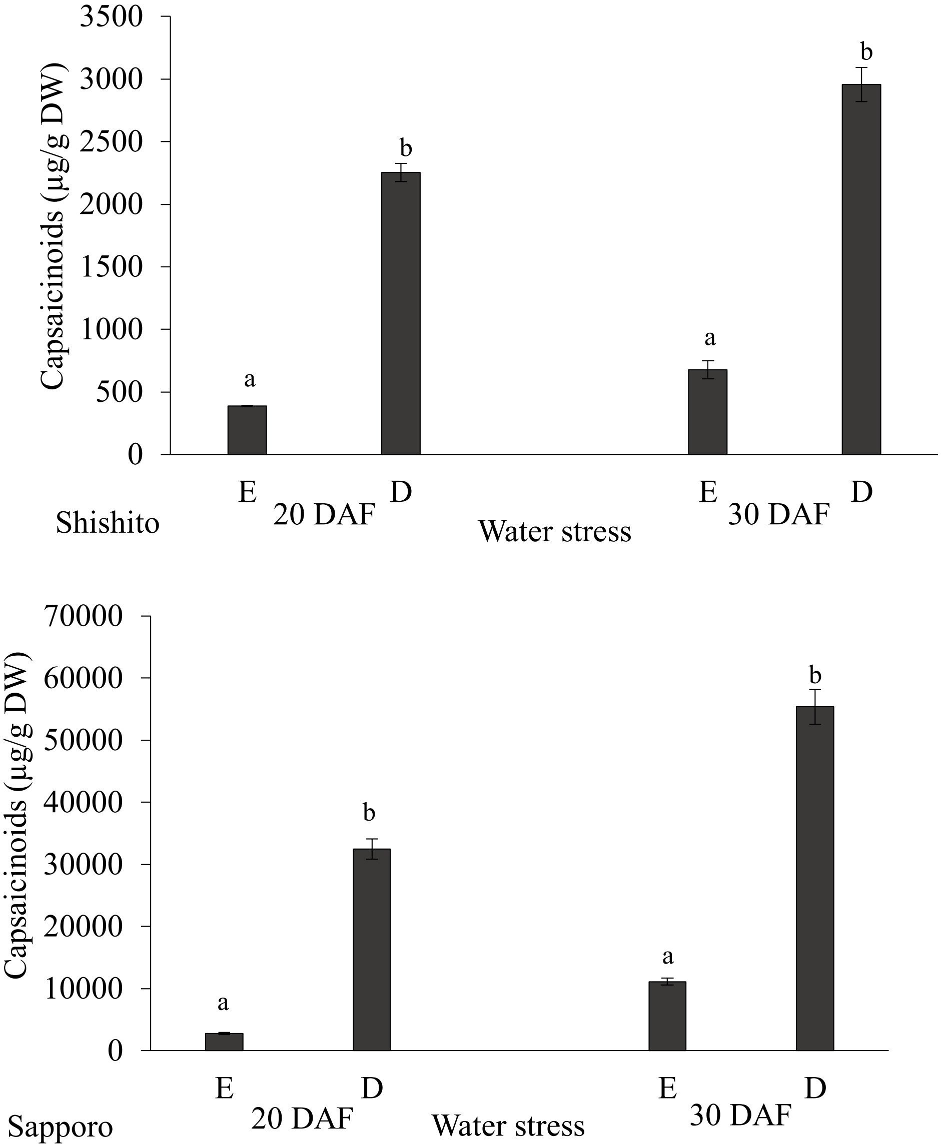


Fig. 4. Capsaicinoid content (µg/g dry weight [DW]) at two stages of fruit maturation in chili peppers ‘Shishito’ and ‘Sapporo’ in 2020. Fruits were sampled at 20 and 30 days after flowering (DAF). Different lowercase letters (a, b) for the same DAF indicate significant differences between treatments (Tukey’s pairwise test, $P < 0.05$). D: drought, E: excess water supply. Error bars indicate the standard error.

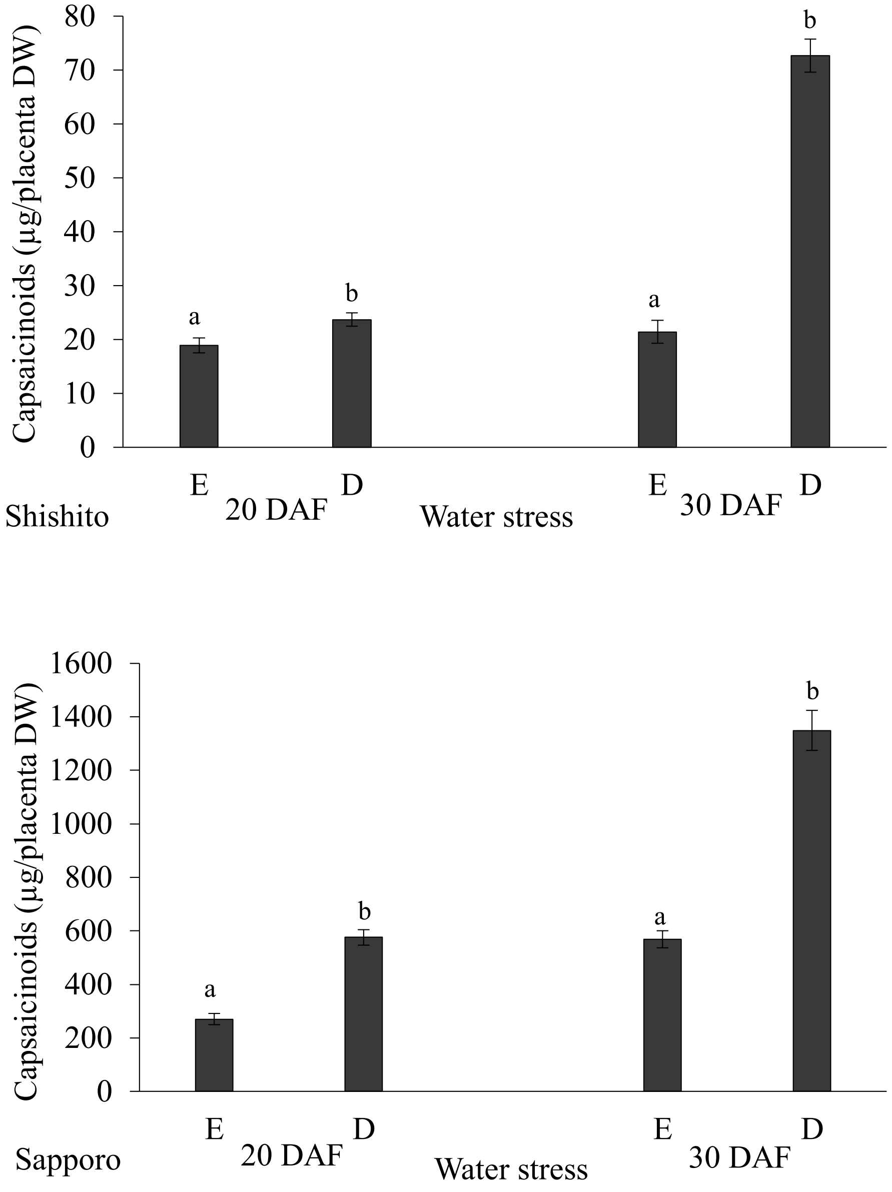


Fig. 5. Capsaicinoid content (µg/placenta dry weight [DW]) at two stages of fruit maturation in chili peppers ‘Shishito’ and ‘Sapporo’ in 2020. Fruits were sampled at 20 and 30 days after flowering (DAF). Different lowercase letters (a, b) for the same DAF indicate significant differences between treatments (Tukey’s pairwise test, $P < 0.05$). D: drought, E: excess water supply. Error bars indicate the standard error.

Expression of capsaicinoid biosynthesis genes

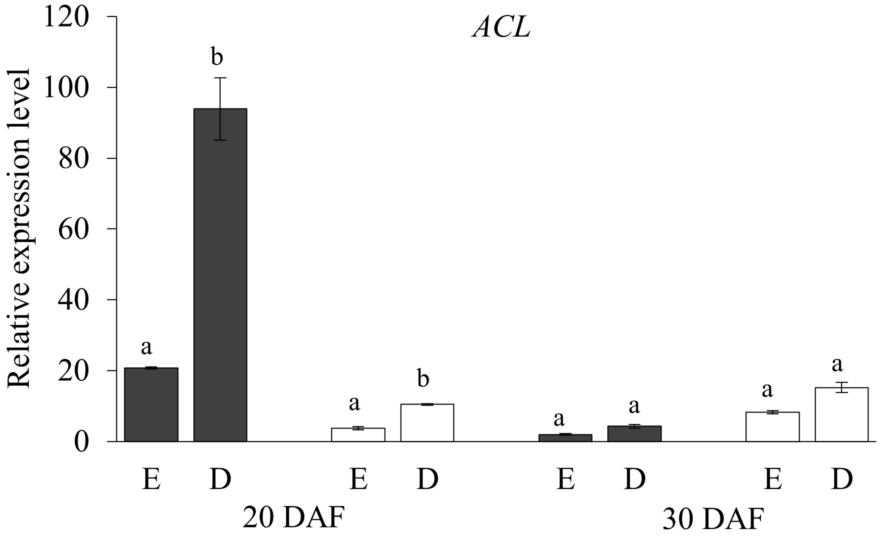
RT-qPCR analysis was conducted for 18 genes involved in the capsaicinoid biosynthesis pathway using three placental septum samples for each treatment and each cultivar. According to the obtained gene expression patterns, I divided the 18 genes into three groups (groups 1, 2, and 3; Fig. 6). The genes in group 1 showed higher gene expression levels in the drought stress condition than in the excess water supply conditions in both cultivars at 20 DAF. The genes in group 1 were *ACL* (*acyl carrier protein*), *pAMT* (*putative aminotransferase*), *Pun1* (*acyltransferase*), *WRKY9*, *CaKR1* (*ketoacyl-ACP reductase*), *CaMYB31, FAT* (*ketoacyl-ACP reductase*), and *KAS I* (*ketoacyl-ACP synthase I*). The *pAMT*, *CaKR1*, and *CaMYB31* genes also showed higher expression levels in the drought stress condition than in the excess water supply condition when harvested 30 DAF in ‘Sapporo’. And *CaMYB31* gene showed higher expression level in drought stress condition than excess water supply condition when harvesting 30 DAF in ‘Shishito’. The genes in group 2 showed higher gene expression levels in the drought stress condition than in the excess water supply condition in only one of the cultivars at 20 DAF. The genes in group 2 were *KAS III* (*ketoacyl-ACP synthase III*), *BCKDH* (*branched-chain α-ketoacid dehydrogenase*), *ACS* (*acyl-CoA synthetase*), *BCAT* (*branched-chain amino acid transferase*), and *4CL* (*4-coumaroyl-CoA ligase*). Among group 2 genes, *KAS III*, *BCKDH*, and *BCAT* were significantly higher levels of expression was showed at 20 DAF in ‘Shishito’, and *BCAT* showed a significantly higher level of expression at 30 DAF in ‘Sapporo’. In addition, *ACS* showed significantly higher levels of expression only at 20 DAF in ‘Sapporo’, and at 30 DAF in ‘Shishito’. The *4CL* gene showed significantly higher expression levels in the drought stress condition than in the excess water stress condition in ‘Sapporo’ at 20 and 30 DAF; however, this was not seen in ‘Shishito’. The genes in group 3 did not show any significant differences in gene expression between the two treatments in either cultivars at all DAF. The genes in group 3 were *PAL* (*phenylalanine ammonia lyase*), *C3H* (*coumaroyl shikimate/quinate 3-hydroxylase*), *HCT* (*hydroxycinnamoyl transferase*), *C4H* (*cinnamate 4-hydroxylase*), and *COMT* (*caffeic acid O-methyltransferase*).

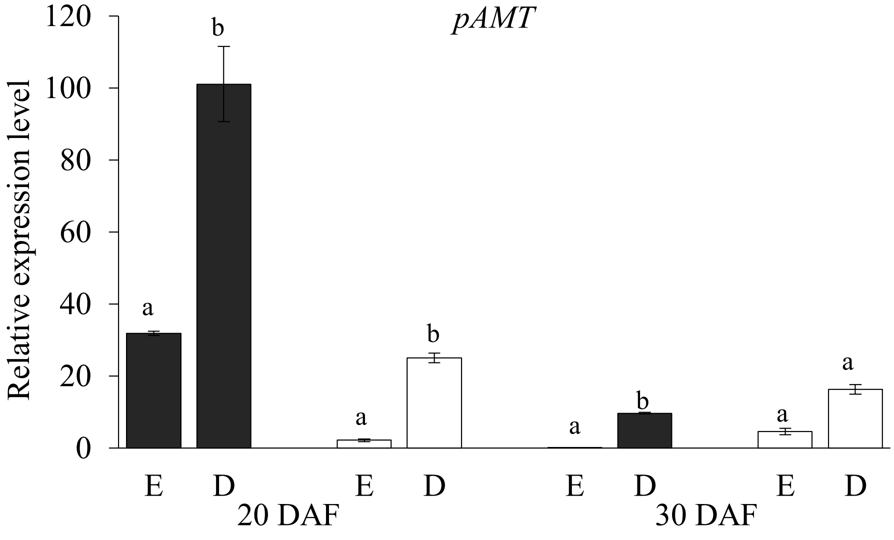
Within the capsaicinoid synthesis pathway, most of the genes belonging to groups 1 and 2 are involved in the branched-chain fatty acid pathway or downstream of the synthesis pathway. Two genes encoding transcriptional factors, *CaMYB31* and *WRKY9,* were assigned to group 1. The genes assigned to group 3 are all involved in the phenylpropanoid synthesis pathway, except for *4CL* and *pAMT,* which are involved downstream of the pathway.

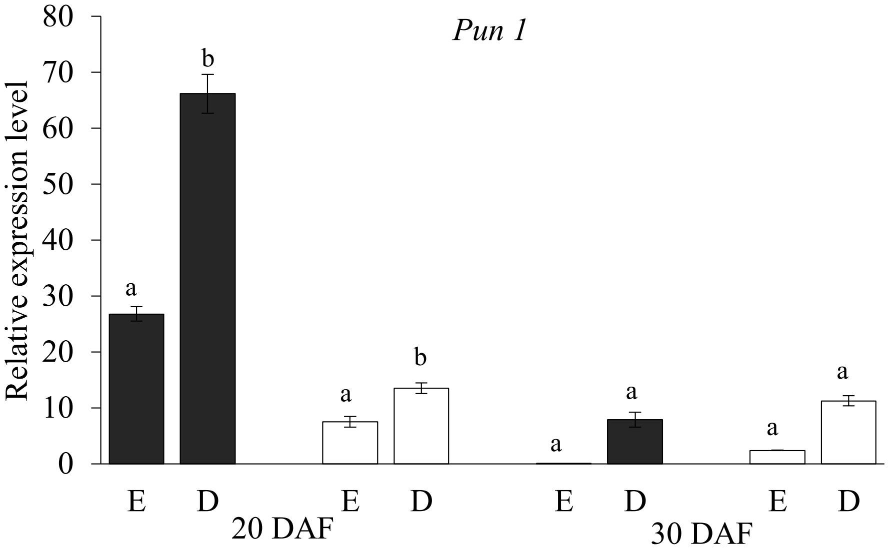
Group 1

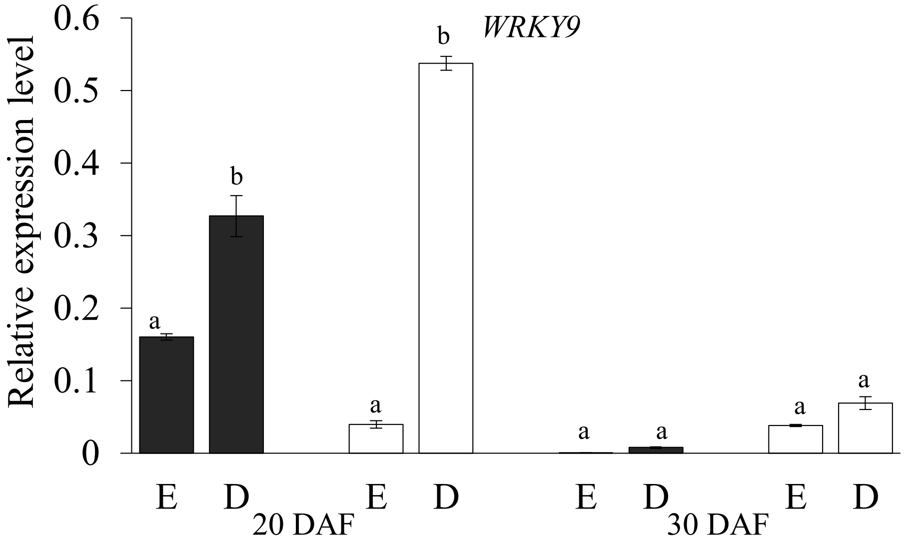
Sapporo ■

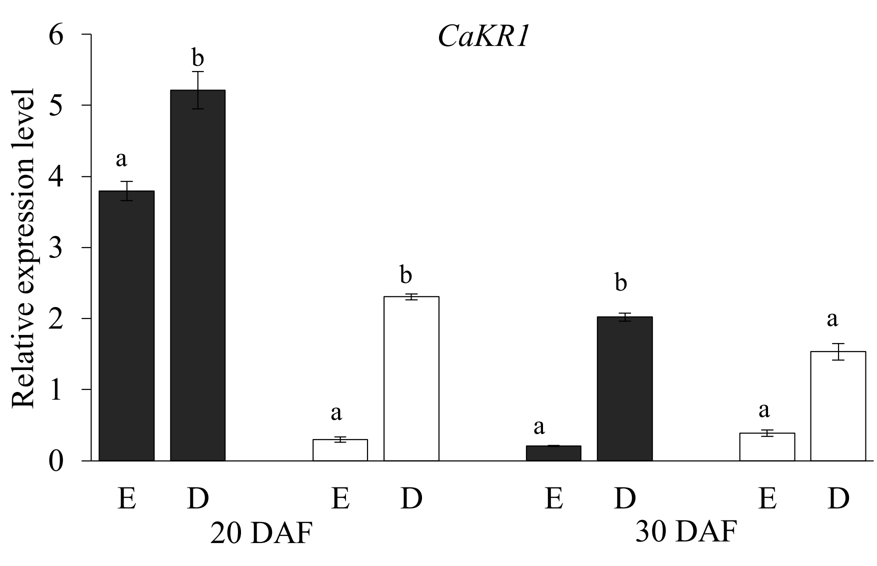
Shishito □

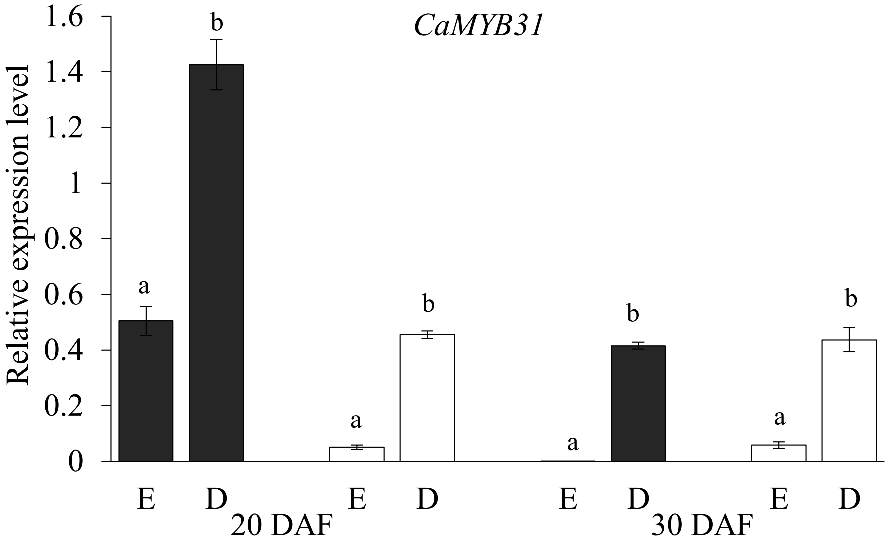


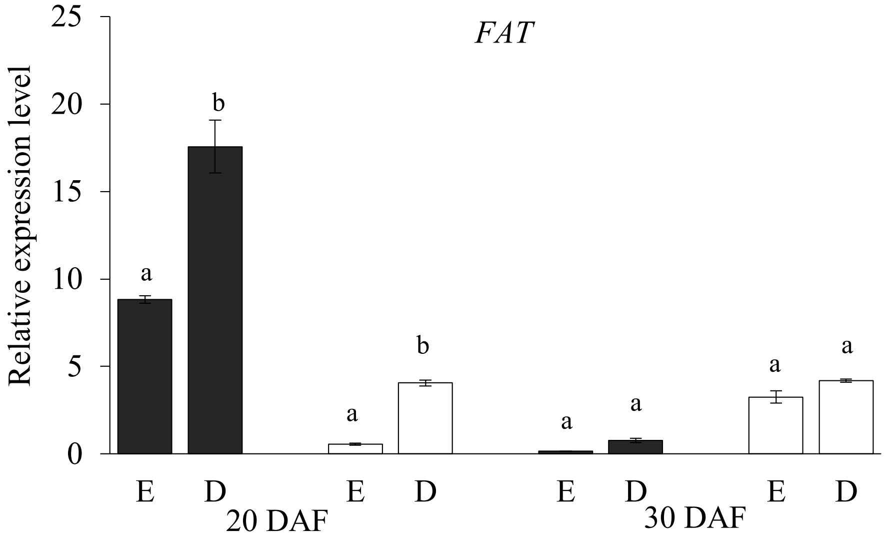


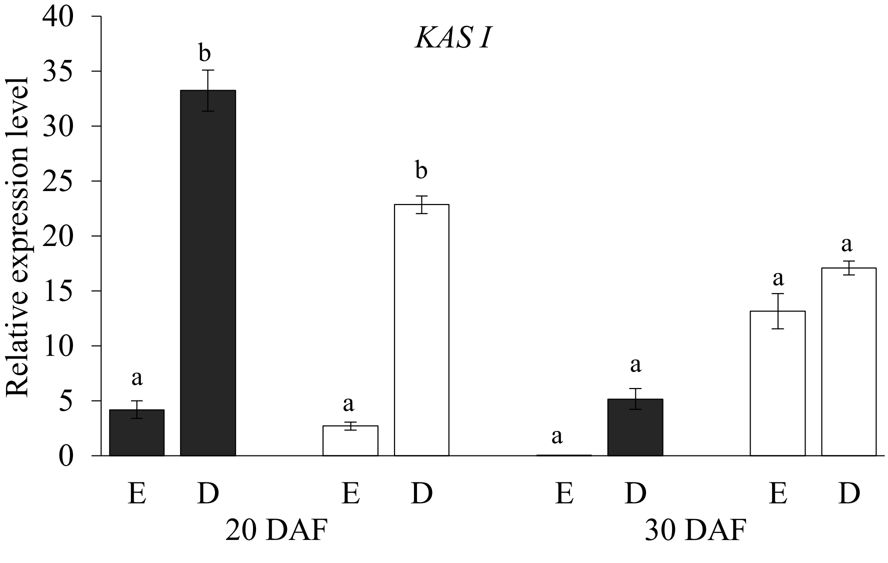

Pun 1
Relative expression level
0
10
20
30
40
50
60
70
80
a
b
a
b
a
a
a
a
E
D
20 DAF
E
D
E
D
30 DAF
E
D


WRKY9
Relative expression level
0
0.1
0.2
0.3
0.4
0.5
0.6
a
b
a
b
a
a
a
a
E
D
20 DAF
E
D
E
D
30 DAF
E
D

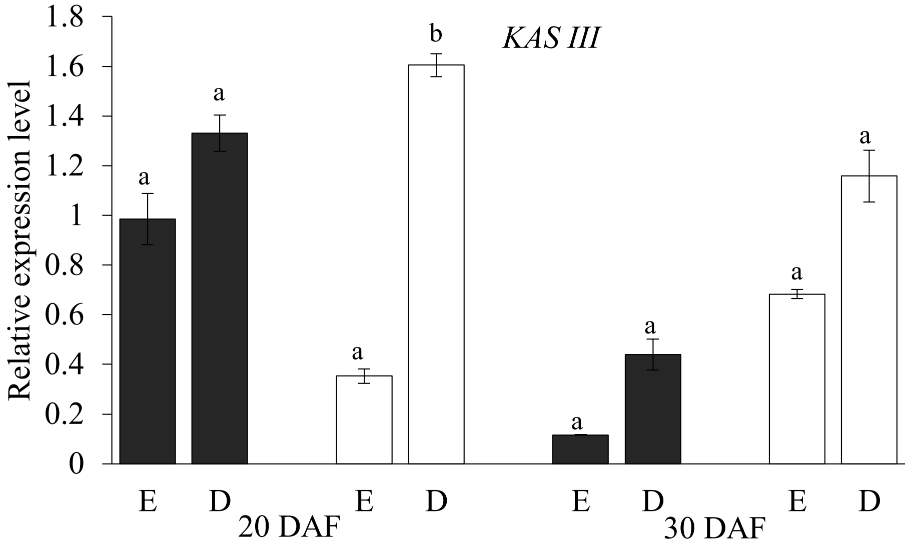

CaKR1
Relative expression level
0
1
2
3
4
5
6
a
b
a
b
a
b
a
a
E
D
E
D
20 DAF
E
D
E
D
30 DAF

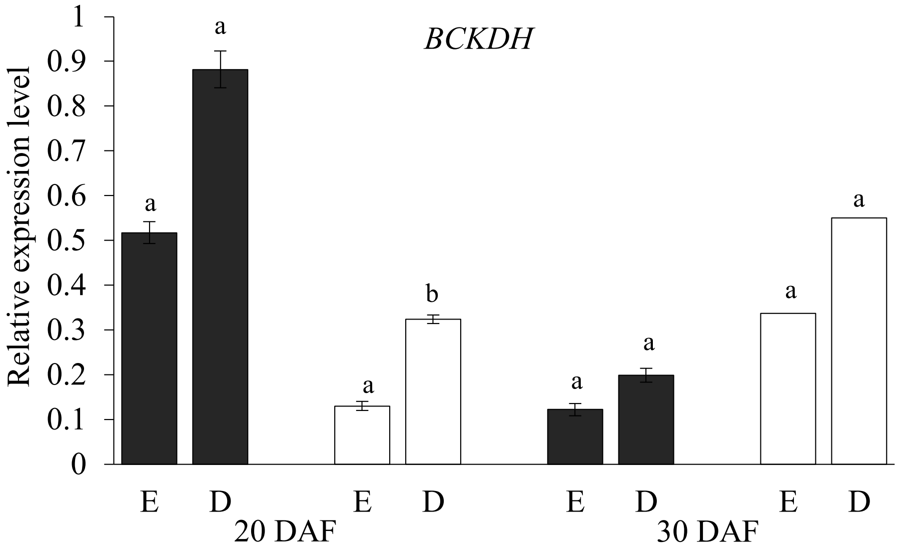

CaMYB31
Relative expression level
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a
b
a
b
a
b
E
D
E
D
E
D
E
D
20 DAF
30 DAF

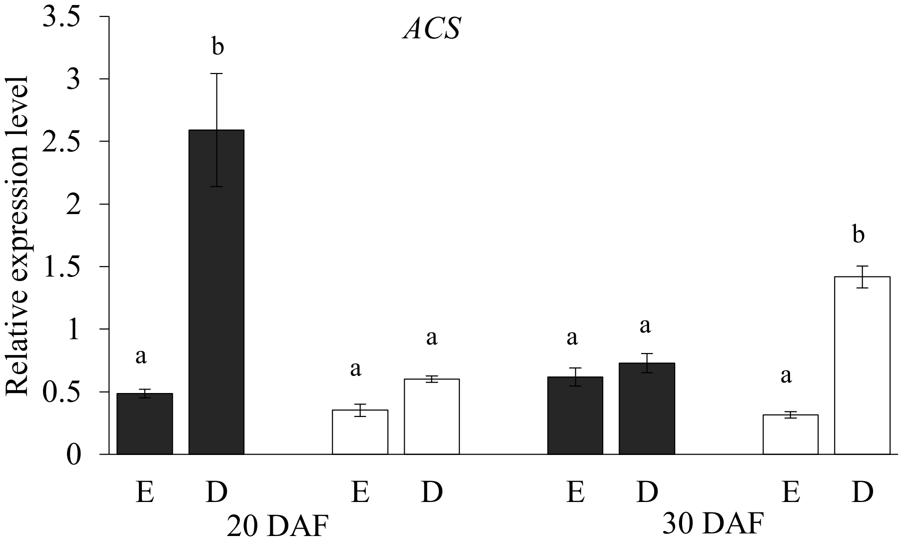

FAT
Relative expression level
25
20
15
10
5
0
a
b
a
b
a
a
a
a
E
D
E
D
E
D
E
D
20 DAF
30 DAF

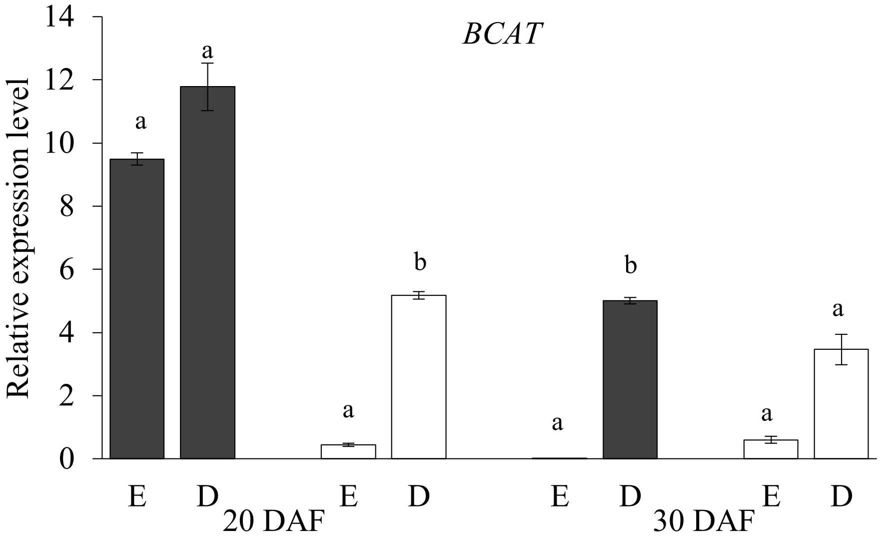

KAS I
Relative expression level
40
35
30
25
20
15
10
5
0
a
b
a
b
a
a
a
a
E
D
E
D
E
D
E
D
20 DAF
30 DAF

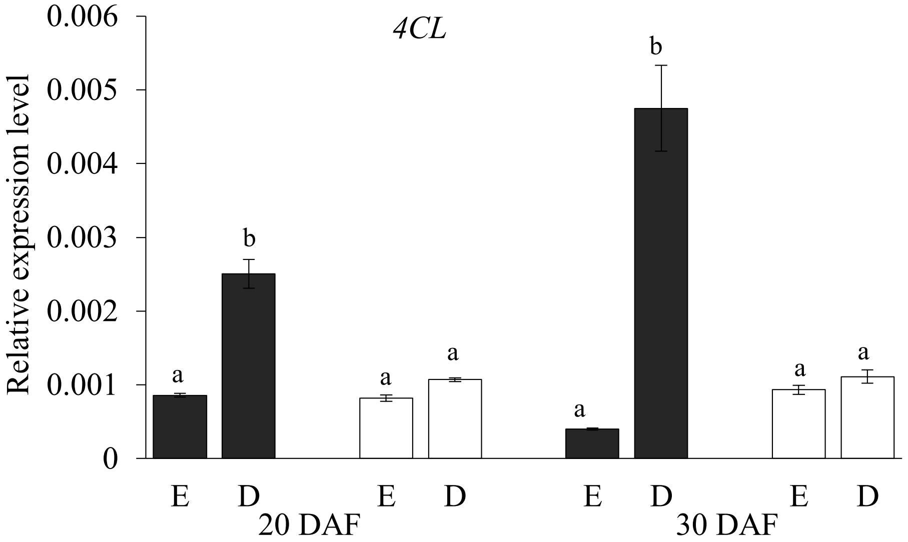
Group 2


KAS III
Relative expression level
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
a
a
a
b
a
a
a
a
E
D
E
D
E
D
E
D
20 DAF
30 DAF

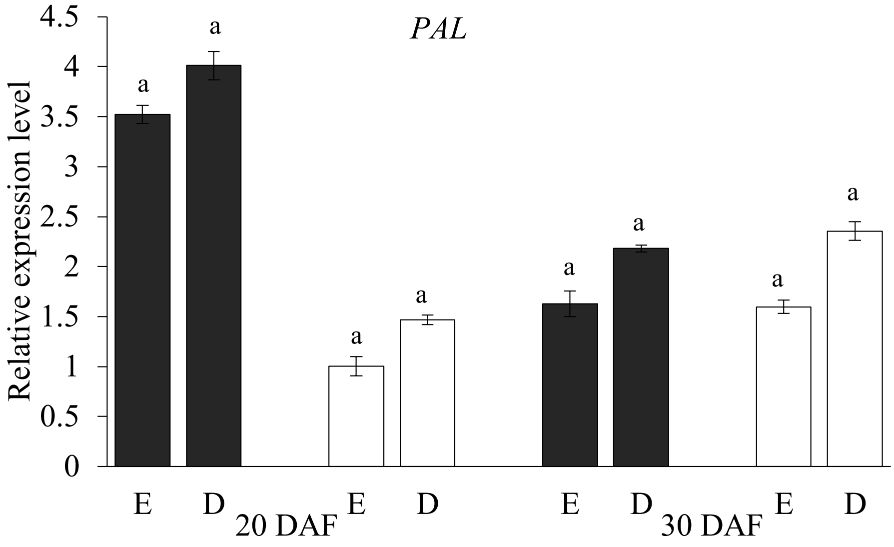

BCKDH
Relative expression level
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
a
a
a
b
a
a
a
a
E
D
E
D
E
D
E
D
20 DAF
30 DAF

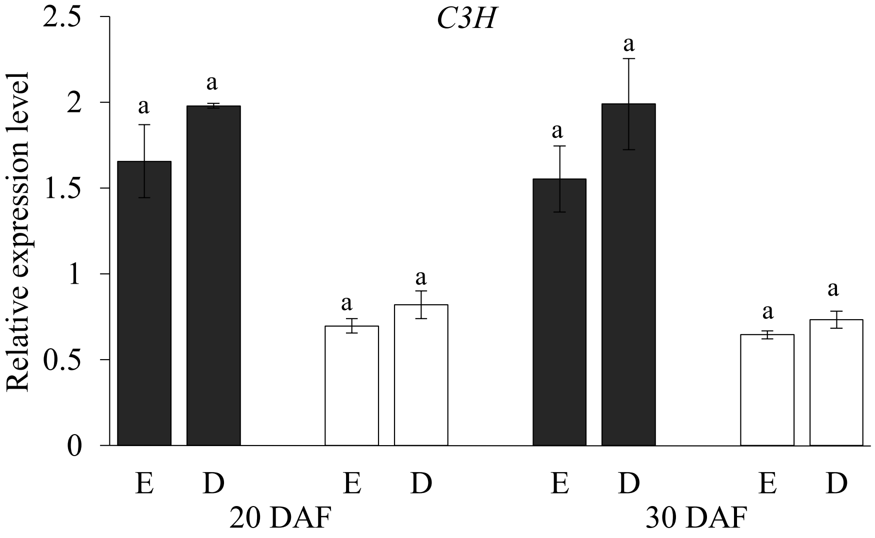

ACS
Relative expression level
0
0.5
1
1.5
2
2.5
3
3.5
a
b
a
a
a
a
a
b
E
D
E
D
E
D
E
D
20 DAF
30 DAF

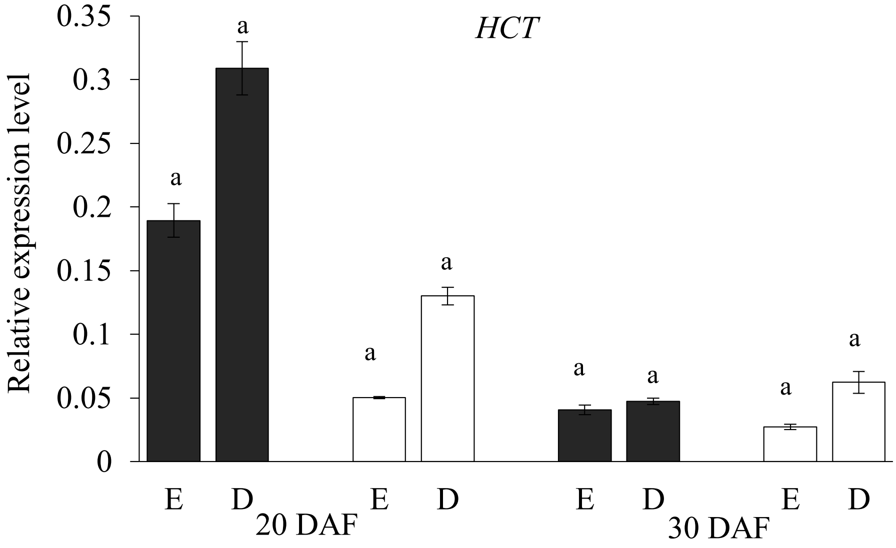

BCAT
Relative expression level
14
12
10
8
6
4
2
0
a
a
a
b
a
b
a
a
E
D
E
D
E
D
E
D
20 DAF
30 DAF

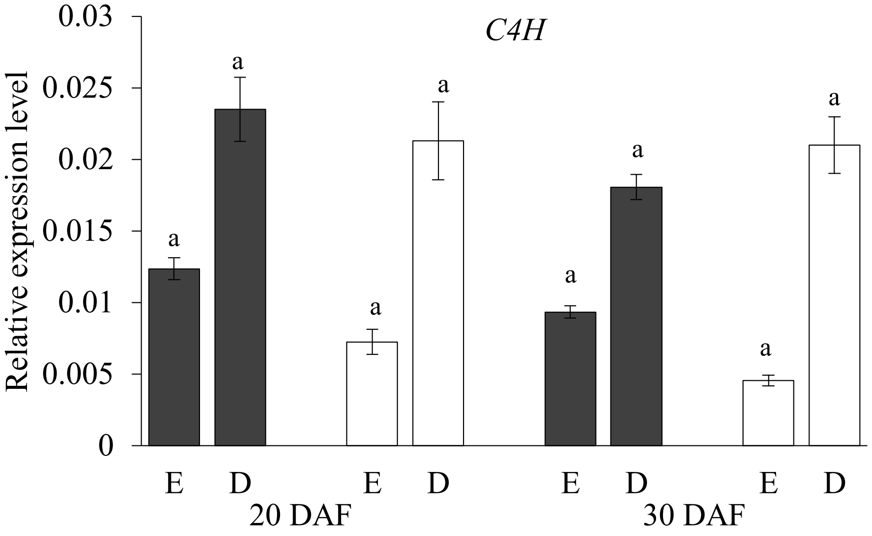

4CL
Relative expression level
0.006
0.005
0.004
0.003
0.002
0.001
0
a
b
a
a
a
b
a
a
E
D
E
D
E
D
E
D
20 DAF
30 DAF

Group 3


PAL
Relative expression level
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
a
a
a
a
a
a
E
D
E
D
E
D
E
D
20 DAF
30 DAF


C3H
Relative expression level
2.5
2
1.5
1
0.5
0
a
a
a
a
a
a
a
a
E
D
E
D
E
D
E
D
20 DAF
30 DAF


HCT
Relative expression level
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a
a
a
a
a
a
a
E
D
E
D
E
D
E
D
20 DAF
30 DAF



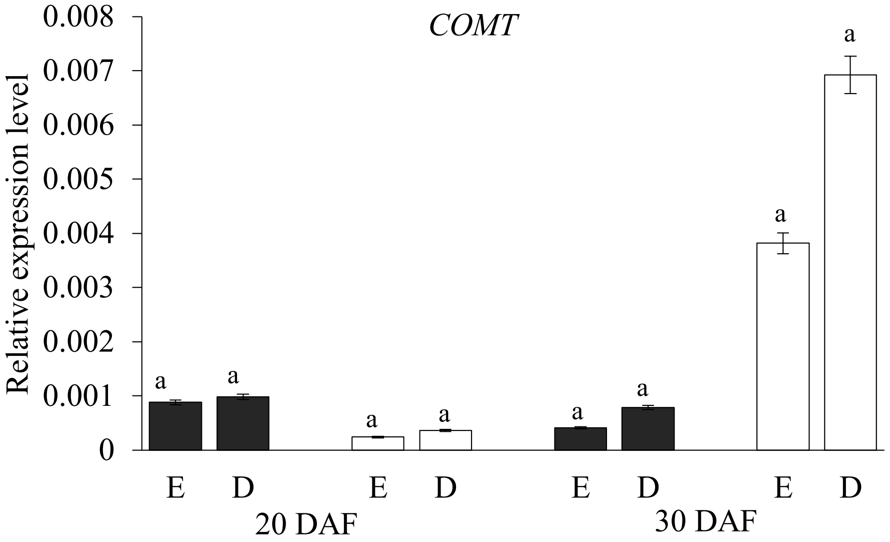


Fig. 6. Relative expression levels of 18 capsaicinoid biosynthesis genes in the placental septum of chili peppers ‘Shishito’ and ‘Sapporo Oonaga Nanban’ cultivated under the drought stress condition (D) and excess water stress condition (E) in 2020. Fruits were sampled at 20 and 30 days after flowering (DAF). Different lowercase letters (a, b) above bars for the same DAF and same cultivar indicate significant differences between treatments (Tukey’s pairwise test, $P < 0.05$). Error bars indicate the standard error (n = 3).

Discussion

The capsaicinoid concentration (capsaicinoid content per unit DW of placental septum) was significantly increased in the drought stress condition in both 'Shishito' and 'Sapporo' (Fig. 4). This result was in line with the my previous findings in chapter II, that the capsaicinoid content in the placental septum increased in the drought stress condition in Japanese chili pepper varieties 'Botankosho' and 'Sapporo' (Rathnayaka et al., 2021). Estrada et al. (1999) also reported that chili fruit was more pungent when grown under drought stress conditions.

I also analyzed the capsaicinoid content per placental septum (µg/placental septum), because the size of the placental septum can affect the capsaicinoid concentration of the whole fruit. The results revealed that the capsaicinoid content per placental septum was also increased in the fruits cultivated under the drought stress condition (Fig. 5), although the DW of the placental septum was quite smaller in the drought stress condition than in the excess water condition. Considering these results, drought stress on the plants appears to increase the pungency levels of the fruit by promoting capsaicinoid synthesis through the activation of genes involved in the capsaicinoid synthesis pathway.

In the present study, RT-qPCR analysis was conducted for 18 capsaicinoid biosynthesis genes, and the expression patterns were compared between the drought stress condition and the excess water condition in 'Shishito' and 'Sapporo'. From the results, I categorized the 18 genes into three groups (groups 1, 2, and 3) based on the expression patterns. *ACL*, *pAMT*, *Pun1*, *WRKY9*, *CaKR1*, *CaMYB31*, *FAT*, and *KAS I* were categorized into group 1, as they were significantly more highly expressed in the drought stress condition than in the excess water stress condition at 20 DAF. These genes are involved in the branched-chain fatty acid pathway, except for *pAMT,* which is involved downstream of the phenylpropanoid pathway, and *Pun1,* which is involved further downstream of the capsaicinoid pathway. According to Stewart et al. (2005, 2007), the most critical gene in capsaicinoid biosynthesis is *Pun1*, which is responsible for the final reaction in the synthesis pathway. The functional loss of *Pun1* due to mutations prevents capsaicinoids synthesis (Stewart et al., 2005). In addition, *pAMT* is also an important gene, as it is required for the production of vanillylamine from vanillin, and mutations in the gene result in reduced capsaicinoid synthesis (Koeda et al., 2014; Lang et al., 2009; Tanaka et al., 2010, 2019). The functional loss of *pAMT* has also been reported to cause a drastic reduction in pungency (Tanaka et al., 2019; Han et al., 2019). The results of this study also showed that the gene expression levels of *Pun1* and *pAMT* were significantly higher in the drought stress condition than in the sufficient irrigation condition at 20 DAF in both cultivars. Moreover, Kondo et al. (2020) mentioned that not only *Pun1* and *pAMT,* but also *KAS I* and *CaMYB31* influenced capsaicinoid biosynthesis in the parthenocarpy-induced fruit placental septum in 'Shishito', resulting in fluctuations in fruit pungency. The genes *KAS III*, *BCKDH, ACS*, *BCAT*, and *4CL*, which had significantly higher expression levels in the drought stress condition than in the excess water condition at 20 DAF in only one of the cultivars, were assigned to group 2. Within the capsaicinoid biosynthesis pathway, the genes in group 2 were found to be involved in the branched-chain fatty acid pathway, similar to the genes in group 1, except for *4CL* and *pAMT,* which are involved in the phenylpropanoid synthesis pathway, *Pun1,* which is involved in the last step of the capsaicinoid pathway, and two genes that encode transcriptional factors. In contrast, genes in group 3, i.e., *PAL*, *C3H*, *HCT*, *C4H*, and *COMT*, showed no significant difference in gene expression level between the drought stress and excess water stress conditions, although they were found to be involved in the phenylpropanoid synthesis pathway. From the results of gene expression analysis, genes assigned to groups 1 and 2 were inferred to likely determine the capsaicinoid content, with the genes in group 1 having a larger effect than those in group 2. According to Kondo et al. (2020), there was no significant difference in the gene expression levels of *ACS*, *BCKDH*, *PAL*, *4CL*, *C4H*, *HCT*, *C3H,* and *COMT* between pungent parthenocarpic fruit and control fruit of the ‘Shishito’ variety. In the present study, there was no significant difference in the gene expression levels of *ACS*, *PAL*, *C4H*, *HCT*, *C3H,* and *COMT* between fruit grown under the drought stress and excess water stress conditions, although a difference was seen for *BCKDH* and *4CL*. Doi et al. (2013) also reported that the expression levels of *pAMT*, *KAS*, *FAT*, *ACL*, *BCAT,* and *Pun 1* increased with increasing pungency in ‘Shishito’ fruits. The present results are in agreement with those results, since five of these genes (all except for *BCAT)* were assigned to group 1, which is considered to strongly affect the pungency. Group 1 also included two genes encoding transcriptional factors, *WRKY9* and *CaMYB31*, which are not involved in the capsaicinoid synthesis pathway. The functional loss of *CaMYB31* has been reported to cause a drastic reduction or the loss of pungency (Han et al., 2019). I also found that the expression levels of *WRKY9* and *CaMYB31* were significantly higher in the drought stress condition than in the excess water condition at 20 DAF in both cultivars. According to Zhu et al. (2019), the *MYB* transcriptional factor encoded by *CaMYB31* directly regulates the expression levels of capsaicinoid biosynthesis genes. Thus, the transcription of group 1 and 2 genes may be controlled by *CaMYB31,* and its upstream gene, *WRKY9,* may regulate the expression levels of genes in the capsaicinoid synthesis pathway. A dual-luciferase reporter assay and chromatin immunoprecipitation-PCR analysis revealed that *CaMYB31* directly targeted a set of capsaicinoid biosynthesis genes to activate their expression (Zhu et al., 2019). It appears that the expression of *CaMYB31* is significantly up regulated in plants under drought stress, and the expression of *MYB* from *CaMYB31* leads to the activation of its target genes, *pAMT*, *Pun 1*, *FAT, KAS*, and *BCAT*. Moreover, genes in group 1 and most of the genes in the group 2 showed similar expression patterns between the drought stress condition and excess water stress condition, indicating that these genes involved in the branched-chain fatty acid pathway are significantly activated by the drought stress condition.

According to Sung et al. (2005), the enzyme activities of *PAL* and *C4H* were significantly higher in the placenta of ‘Beauty Zest’ hot chili peppers (*C. annuum*) from water-deficient plants than in the controls. Moreover, Phimchan et al. (2014) also mentioned that the enzyme activities of *PAL* and *C4H* were higher in drought-stressed plants than in non-drought-stressed plants. However, according to the present results, the *PAL* and *C4H* expression levels were not significantly higher in the drought stress condition. According to Liu et al. (2015), genes involved in the phenylpropanoid pathway, which were assigned to group 3 in the present study (*PAL*, *C3H*, *HCT*, *C4H*, and *COMT*), are also responsible for the biosynthesis of secondary metabolites, such as phenolic acid, flavonoids, and lignin. Therefore, it is assumed that there was no significant difference in the gene expression level of these genes between the stressed and non-stressed conditions in both cultivars and at all DAF. In addition, the expression patterns of group 3 genes that are involved in the phenylpropanoid pathway were identical to those reported by Kondo et al. (2020) for artificial parthenocarpic ‘Shishito’ fruit.

Of note, the expression level of most of the genes in all groups was higher in ‘Sapporo’ than in ‘Shishito’ at 20 DAF. This result is reasonable, because ‘Sapporo’ is a pungent variety, and it would thus be expected to synthesize more capsaicinoids than ‘Shishito’, which is a very low pungent variety.

In conclusion, my results indicated that the stress on the plants increased the capsaicinoid content of the fruit by promoting capsaicinoid synthesis in the placental septum. I found that 18 genes involved in the capsaicinoid biosynthesis pathway, especially the genes in groups 1 and 2, were responsible for the increased pungency of fruit from drought-stressed plants. However, further experiments using several other stress conditions (e.g., salinity, high temperature, and soil fertility) are needed to confirm the behavior of the genes responsible for capsaicinoid synthesis under conditions of stress. In addition, the key genes that are responsible for promoting capsaicinoid synthesis under conditions of stress must be further explored.

Chapter VI

General Discussion

Crop growth and development are constantly influenced by environmental conditions, such as stresses, which are the most important yield reducing factors in the world (Dennis, 2000). Various environmental stresses including high winds, extreme temperatures, soil salinity, drought, and flood have affected the production and cultivation of agricultural crops, among these, drought stress and soil salinity are the most devastating environmental stresses, and cause major reductions in cultivated land area, crop productivity, and quality (Yamaguchi and Blumwald, 2005; Shahbaz and Ashraf, 2013). Drought stress is considered the main limiting factor of crop performance and a threat to successful crop production. It seems that 25% of all agricultural lands are limited because of drought stress. Soil salinization is the most important environmental stress next to drought in many parts of the world. It has been estimated that 20% of total cultivated and 33% of irrigated agricultural lands worldwide are afflicted by high salinity. Furthermore, salinized areas are increasing at a rate of 10% annually for various reasons, including low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices. It has been estimated that more than 50% of arable land would be salinized by the year 2050 (Jamil et al., 2011). Therefore, experiments on the effect of drought stress and salinity stress conditions on crops are very important considering the increasing population and increasing demand. Although chili pepper is one of the main spices in the world, there have been limited experiments on the effects of stress to chili pepper, especially with regards to its taste components.

In Chapter II, the effect of drought stress on the taste component of chili pepper fruits was investigated. The results revealed that drought stress is significantly effective for increasing Brix, total sugar content, glutamic acid content, and capsaicinoids in the fruits. Moreover, it was revealed that delayed harvesting also significantly increased Brix, total sugar content, glutamic acid content, and capsaicinoids in the chili pepper. According to Yin et al. (2010) and Nuriddin et al. (2003), water deficit may benefit tomato fruit quality due to the increased levels of total soluble solids (sugars, amino acids, and organic acids), which are the major compounds accumulating in the fruit. In the case of tomato, total sugar and Brix were increased by drought condition (Nahar and Gretzmacher, 2002; Sadamasa, 2008). The present experimental results also agreed for chili pepper with increasing total sugar and Brix percentage when plants were in the drought stress condition. Estrada et al. (1999) also found that capsaicinoid content in chili pepper fruits increased in drought-stressed plants and the increase of capsaicinoid content in low-medium-pungent-cultivars under the drought stress condition was significant (Paongpetch et al., 2012). Capsaicinoid content in pungent cultivar and placental septum also increased when the plant was under the drought stress condition in the present experiment.

Following the results obtained in Chapter III, the effect of salinity stress on the taste component of the chili pepper was investigated. The results revealed that salinity stress significantly increased Brix, total sugar content, glutamic acid content and capsaicinoids in chili pepper. Moreover, the experiment revealed that delayed harvesting also significantly increased Brix, total sugar content, glutamic acid content and capsaicinoids in chili pepper. This finding agrees with reports suggesting that salt stress increases the amount of soluble sugars of rice (Pattanagul and Thitisaksakul, 2008; Siringam et al., 2011; Amirjani, 2011), Chenopodium (Prado et al., 2000), and sugar beet (Khavari et al., 2008). In fact, it is believed that the accumulation of total soluble sugars is a common phenomenon under stress conditions (William et al., 2000; Murakeozy et al., 2003). The above-mentioned results agree with Rafael et al. (2014) that capsaicin and dihydrocapsaicin levels in Jalapeno peppers had the highest value under high salinity treatments.

According to the results of Chapters II and III, stress conditions (drought and salinity) caused increased taste components in chili pepper. These results were positive points when chili pepper are cultivated in stress affected areas; however, it is important to determine the effects of stress conditions on yield and plant physiology. Therefore, in Chapter IV, an experiment was conducted to determine the effect of stress conditions on Brix, capsaicinoid content, yield, plant growth and fruit parameters. Both stress conditions caused significantly lower number of fruits, fruit weight, total yield, number of leaves, number of branches, and plant height. According to the overall results in Chapter IV, plant growth parameters and fruit parameters were negatively sensitive to water stress and salinity stress. Zhani et al. (2012) also found that salinity stress significantly impacted the plant height, and the fresh and dry biomass of the chili pepper. In both stress conditions, the Brix value significantly increased giving similar results even the fruit weight/fruit size decreased under the stress condition. This means that the total capacity for photosynthesis should be increased by the stress condition. The highest content of capsaicinoids in placental septum was observed in the drought and excess salinity stress treatments. According to analyses of capsaicinoid, the higher amount of capsaicinoids seen in stress conditions was not because it was concentrated into a smaller fruit area, but due to accelerated capsaicinoid synthesis because of stress conditions.

Therefore, the necessity of elucidating genetic mechanisms by conducting expression analyses of genes involved in synthesis and accumulation of taste components when plants were under stress conditions was identified.

According to the findings of Chapters II, III, and IV, even if the amount of taste components was increased by the stress condition, it adversely affected plant yield, plant growth, and fruit parameters. In addition, we found that taste components were increased maybe because of the accelerated synthesis mechanisms due to the stress condition. Therefore, in Chapter V, an experiment was conducted to determine the drought stress-induced increase of pungency and expression of capsaicinoid biosynthesis genes in chili pepper. Throughout the gene expression analysis for 18 capsaicinoid biosynthesis genes, the results showed that most of the capsaicinoid biosynthesis genes belonged to a branched chain fatty acid pathway that was significantly highly expressed in the drought stress condition and at 20 DAF. However, most genes belonging to the phenylpropanoid pathway did not show significant differences in gene expression between stressed and not stressed chili peppers.

The present studies clarified the relationships among major environmental stress conditions such as drought stress and salinity stress conditions that positively affect the taste components (sugars, glutamic acid, and capsaicinoids), and negatively affect parameters related to fruit (total yield, number of fruits and fruit weight) and plant growth parameters (number of leaves, number of branches, and plant height) in chili pepper fruit. Also, we have clarified the highest amount of capsaicinoid content in the stress conditions happened due to accelerating the capsaicinoid synthesis capacity in the placental septum. However, to better understand how environmental factors affect the changes in the contents of taste components, further investigations are necessary. Therefore, it is necessary to carry out further experiments using several stress conditions (salinity, high temperature, soil fertility, etc.) and to clarify the behavior of the genes responsible for capsaicinoid synthesis under stress conditions.

Acknowledgement

I would like to thanks the Dr. Tomoo Maeda in Hirosaki University, the Gene bank of NARO and six farmers' groups in Nagano prefecture, for providing seeds of 'Shimizumri Namna', 'Sanpou Ooamanaga' and the other local chili pepper varieties, respectively.

References

Adams, P. and L. C. Ho. 1992. The susceptibility of modern tomato cultivars to blossom-end rot in relation to salinity. J. Hort. Sci. 67: 827-839.

Amirjani, M. R. 2011. Effect of salinity stress on growth, sugar content, pigments and enzyme activity of rice. Inter. J. Bot. 7: 73-81.

Andrew, J. 1995. Peppers-The domesticated capsicums. New Edition. University of Texas Press, Austin, TX, USA.

Arce-Rodríguez, M. L. and N. Ochoa-Alejo. 2019. Biochemistry and molecular biology of capsaicinoids biosynthesis: recent advances and perspectives. Plant Cell Rep. 38: 1017-1030.

Arrowsmith, S., T. P. Egan, J. F. Meekins, D. Powers and M. Metcalfe. 2012. Effects of salinity stress on capsaicin content, growth, and fluorescence in a Jalapeno cultivar of *Capsicum annuum* (Solanaceae). J. BIOS 83:1-7.

Azuma, R., N. Ito, N. Nakayama, R. Suwa, N. T. Nguyen, J. A. Larrinaga-Mayoral, M. Esaka, H. Fujiyama and H. Saneoka. 2010. Fruits are more sensitive to salinity than leaves and stems in pepper plants (*Capsicum annuum* L.). Hort. Sci. 125: 171-178.

Bakr, J., H. G. Daood, Z. Pek, L. Helyes and K. Posta. 2016. Yield and quality of mycorrhized processing tomato under water scarcity. Appl. Ecol. Environ. Res. 15: 401-413.

Barchenger, D. W. and P. W. Bosland. 2019. Wild chili pepper (*Capsicum* spp.) of North America, P. 225-242.

Bolarin, M. C., M. T. Estan, M. Caro, R. Romero-Aranda and J. Cuartero. 2001. Relationship between tomato fruit growth and fruit osmotic potential under salinity. Plant Sci. 160: 1153-1159.

Bosland, P. W. and E. J. Votava. 2002. Peppers: Vegetable and spice *Capsicum.* CABI, New York, NY.

Brunner, I. and C. Sperisen. 2013. Aluminum exclusion and aluminum tolerance in woody plants. Front. Plant Sci. 4: 1-12.

Butt, M., C. M. Ayyub, M. Amjad and R. Ahmad. 2016. Proline application enhances growth of chili by improving physiological and biochemical attributes under salt stress. Pak. J. Agri. Sci. 53: 43-49.

Channabasavann, A. S. and R. A. Setty. 2000. Effect of different irrigation intervals on sweet pepper. South Indian. Hort. 39: 296-296.

Chretien, S., A. Gosselin and M. Dorais. 2000. High electrical conductivity and radiation-based water management improve fruit quality of greenhouse tomatoes grown in rockwool. Hort. Sci. 35: 627-631.

Dennis, B. E. and W. P. Bruening. 2000. Potential of early maturing soybean cultivars in late plantings. Agro. J. 92: 532-537.

Doi, M., S. Matsubara and S. Koeda. 2013. Expression of capsaicin synthesis-related genes in *Capsicum annuum* L. Shishitou fruits. Hort. Res. (Japan) 12 (Suppl. 1): 321 (In Japanese).

Drake, D. R. and I. A. Ungar. 1989. The effects of salinity, nitrogen level, and population density on the survival, growth, and reproduction of *Atriplex triangularis* (Chenopodiaceae). American Journal of Botany 76: 1125-1135.

Ehret, D. L. and L. C. Ho. 1986. The effects of salinity on dry matter partitioning and fruit growth in tomatoes grown in nutrient film culture. J. Hort. Sci. 61: 361-367.

Estrada, B., F. Pomar, J. Diaz, F. Merino and M. A. Bernal. 1997. Evolution of capsaicinoids in *Capsicum annuum* L. var. *annuum* cv. Padron fruit at different growth stages after flowering. Capsicum Eggplant Newsl. 16: 60-63.

Estrada, B., F. Pomar, J. Diaz, F. Merino and M. A. Bernal. 1999. Pungency levels in fruit of the Padron pepper with different water supply. Sci. Hort. 81: 385-396.

FAOSTAT, 2014. "Chili production in 2014; Crops/World Regions/Production Quantity/Chilies and Peppers, Green and Dried from pick lists". UN Food and Agriculture Organization, Statistics Division. 2017. Retrieved 7 August 2017.

Greenway, H. and R. Munns. 1980. Mechanisms of salt tolerance in non-halophytes. Annual Review of Plant Physiology and Plant Molecular Biology. 31: 149-190.

Han, K., S. Jang, J. H. Lee, D. G. Lew, J. K. Kwon and B. C. Kang. 2019. A MYB transcription factor is a candiDAFe to control pungency in *Capsicum annuum*. Theor. Appl. Genet. 132: 1235-1246.

Han, K., H. Lee, N. Ro, O. Hur, J. Lee, J. Kwon and B. Kang. 2018. QTL mapping and GWAS reveal candidate genes controlling capsaicinoid content in *Capsicum*. Plant Biotechnol. J. 16: 1546–1558.

Harvell, K. P. and P. W. Bosland. 1997. The environment produces a significant effect on pungency of chilies. Hort. Sci. 32: 1292-2014.

Hatakeyama, K., M. Kitazawa, Y. Park, K. Nemoto, H. Kitamura and K. Matsushima. 2017. Evaluation of taste components contents in *Capsicum annuum*. Hort. Sci. 16 (Extra issue 1): 83 (In Japanese).

Hayata, Y., T. Tabe, S. Kondo and K. Inoue. 1998. The effects of water stress on the growth, sugar and nitrogen content of cherry tomato fruit. J. Japan. Soc. Hort. Sci. 67: 759-766 (In Japanese).

Horie, H. 2009. Analysis for the taste components in various vegetables by capillary electrophoresis. Bunseki kagaku. 58: 1063-1066 (In Japanese with English summary).

Iwai, K., T. Suzuki and H. Fujiwake. 1979. Formation and accumulation of pungent principle of hot pepper fruits, capsaicin and its analogues, in *Capsicum annuum* var. *annuum* cv. Karayatsubusa at different growth stages after flowering. Agri. Biol. Chem. 43: 2493-2498.

Jamez, R. E., E. Rada and C. Garcia-Nunez. 1999. The effect of irrigation frequency on water and carbon relations in three cultivars of sweet pepper (*Capsicum chinense* Jacq.), in a tropical semiarid region. Sci. Hort. 81: 301-308.

Jamil, A., S. Riaz, M. Ashraf and M. R. Foolad. 2011. Gene expression profiling of plants under salt stress. Crit. Rev. Plant Sci. 30: 435-458.

Kader, A. A., M. A. Stevens, M. A. Holton, L. L. Morris and M. Agazi. 1977 Effect of fruit ripeness when picked on flavor and composition in fresh market tomatoes. J. Amer. Soc. Hort. Sci. 102: 724-731.

Khavari, N. R. A., F. Najafi and S. N. Khavari. 2008. Growth and some physiological parameters of four sugar beet (*Sugar vulgaris* L.) cultivars as affected by salinity. Pak. J. Biol. Sci. 11: 1390-1394.

Koeda, S., K. Sato, H. Saito, A. J. Nagano, M. Yasygi, H. Kudoh and Y. Tanaka. 2019. Mutation in the putative ketoacyl-ACP reductase *CaKR1* induces loss of pungency in *Capsicum*. Theor. Appl. Genet. 132: 65-80.

Koeda, S., K. Sato, K. Tomi, Y. Tanaka, R. Takisawa, M. Hosokawa, M. Doi, T. Nakazaki and A. Kitajima. 2014. Analysis of non-pungency, aroma, and origin of a *Capsicum chinense* cultivar from a Caribbean island. J. Japan. Soc. Hort. Sci. 83: 244-251.

Kondo, F., K. Hatakeyama, A. Sakai, M. Minami, K. Nemato and K. Matsushima. 2020. Parthenocarpy induced fluctuations in pungency and expression of capsaicinoid biosynthesis genes in a Japanese pungency-variable sweet chili pepper 'Shishito' (*Capsicum annuum*). J. Japan. Soc. Hort. Sci. (In print)

Kosuge, S. and Y. Inagaki. 1962. Studies on the pungent principles of red pepper. Part XI. Determination and contents of the two pungent principles. J. Agric. Chem. Soc. 36: 251-254 (In Japanese).

Kraft, K. H., C. H. Brown, G. P. Nabhan, E. Luedeling, J. de J. L. Ruiz, G. C. d'Eeckenbrugge, R. J. Hijmans and P. Gepts. 2014. Multiple lines of evidence for the origin of domesticated chili pepper *Capsicum annuum* in Mexico. Proc. Natl. Acad. Sci. U.S.A. 111: 6165-6170.

Kramer, P. J. 1969. Plant and water relationships. A modern synthesis. McGraw-hill, New York.

Kramer, P. J. 1980. Drought stress and the origin of adaptation. In: Turner, N.C. and P.J. Kramer (eds.). Adaptation of plants to water and high temperature stress. Wiley Inter science, New York, NY.

Lang, Y., H. Kisaka, R. Sugiyama, K. Nomura, A. Morita, T. Watanabe, Y. Tanaka, S. Yanzawa and T. Miwa. 2009. Functional loss of *pAMT* results in biosynthesis of capsinoids, capsaicinoid analogs, in *Capsicum annuum* cv. CH-19 sweet. Plant J. 59: 953-961.

Li, J., L. Liu, Y. Bai, P. Zhang, R. Flinkers, Y. Du, R. G. F. Visser and A. W. van Heusden. 2011. Seedling salt tolerance in tomato. Euphytica 178: 403.

Li, Y. L., C. Stanghellini and H. Challa. 2001. Effect of electrical conductivity and transpiration on production of greenhouse tomato (*Lycopersicon esculentum* L.). Hort. Sci. 88: 11-29.

Liu, J., A. Osbourn and P. Ma. 2015. MYB transcription factors as regulators of phenylpropanoid metabolism in plants. Mol. Plant 8: 689-708.

Madhumathy, A. P., A. A. Aivazi and V. A. Vijayan. 2007. Larvicidal efficacy of *Capsicum annum* against *Anopheles stephensi* and *Culex quinquefasciatus*. J. Vet. Borne. Dis. 44: 223-226.

Minami, M., M. Toyoda, T. Inoue, K. Nemoto and A. Ujihara. 1998. Changes of capsaicinoid contents during maturing stage in chili pepper (*Capsicum* spp.). J. Fac. Agric. Shinshu Univ. 35: 45-49 (In Japanese).

Murakeozy, E. P., Z. Nagy, C. Duhaze, A. Bouchereau and Z. Tuba. 2003. Seasonal changes in the levels of compatible osmolytes in three halophytic species of inland saline vegetation in Hungry. J. Plant Physiol. 160: 395-401.

Nahar, K. and R. Gretzmacher. 2002. Effect of water stress on nutrient uptake, yield and quality of tomato under subtropical conditions. Die Bodenkultur. Res. 53: 45-51.

Najet, G., L. B. Yahia, B. Lachiheb and A. Ferchichi. 2016. Salt response in pepper (*Capsicum annuum* L.): Components of photosynthesis inhibition, proline accumulation, and K^+/Na^+ selectivity. J. Aridland Agric. 2: 1-12.

Nielsen, T. H., H. C. Skjaerbaek and P. Karlsen. 1991. Carbohydrate metabolism during fruit development in sweet pepper (*Capsicum annuum*) plants. Physiologia Plantarum. 82: 311-319.

Niu, G. and D. S. Rodriguez. 2010. Salinity and soil type effects on emergence and growth of pepper seedlings. Hort. Sci. 45: 1265-1269.

Niu, G., D. S. Rodriguez, E. Call, P. W. Bosland, A. Ulery and E. Acosta. 2010. Responses of eight chili peppers to saline water irrigation. Sci. Hort. 126: 215-222.

Nonaka, T., K. Matsushima, M. Minami, K. Nemoto and Y. Hamauzu. 2012. Changes in antioxidant and taste compounds in 'Botankoshou' (*Capsicum annuum* L.) fruit, a local chili pepper variety from Nagano during stage. Hort. Res. 11: 379-385 (In Japanese with English summary).

Nuriddin, M. M., C. A. Madramootoo and G. T. Dodds. 2003. Effects of water stress at different growth stages on greenhouse tomato yield and quality. Hort. Sci. 38: 1389-1393.

Okano, K., Y. Nakano, S. Watanabe and T. Ikdeda. 2002. Control of fruit quality by salinity stress at various fruit development stages of single-truss tomato grown in hydroponics. Environ. Con. Biol. 40: 375-382 (In Japanese with English summary).

Ortiz, R., J. Crossa, M. Vargas and J. Izquierdo. 2007. Studying the effect of environmental variables on the genotype × environment interaction of tomato. Euphytica 153:119-134.

Othman, Z. A. A., Y. B. H. Ahmed, M. A. Habila and A. A. Ghafar. 2011. Determination of capsaicin and dihydrocapsaicin in *capsicum* fruit samples using high performance liquid chromatography. Molecules. 16*:* 8919-8929.

Paongpetch, P., S. techawongstien, S. Chanthai and P. W. Bosland. 2012. Impact of drought stress on the accumulation of capsaicinoids in capsicum cultivars with different initial capsaicinoid levels. Hort. Sci. 47: 1204-1209.

Pattanagul, W. and M. Thitisaksakul. 2008. Effect of salinity stress on growth and carbohydrate metabolism in three rice (*Oryza sativa* L.) cultivars differing in salinity tolerance. Indian J. Exp. Biol. 46: 736-742.

Pena, R. and J. Hughes. 2007. Improving vegetable productivity in a variable and changing climate. SAT. E. J. 4: 1-22.

Perry, L., R. Dickau, S. Zarrillo, I. Holst, D. M. Pearsall, D. R. Piperno, M. J. Berman, R. G. Cooke, K. Rademaker, A. J. Ranere, J. S. Raymond, D. H. Sandweiss, F. Scaramelli, K. Tarble and J. A. Zeidler. 2007. Starch fossils and the domestication and dispersal of chili peppers (*Capsicum* spp. L.) in the America. Science. 315: 986-988.

Phimchan, P., S. Chanthai, P. W. Bosland and S. Techawongsten. 2014. Enzymatic changes in phenylalanine ammonia-lyase, cinnamic-4-hyroxylase, capsaicin synthase, and peroxidase activies in Capsicum unde drought stress. J. Agric. Food Chem. 62: 7057-7062.

Prado, F. E., C. Boero, M. Gallardo and J. A. Gonzalez. 2000. Effect of NaCl on germination, growth, and soluble sugar content in *Chenopodium quinoa* Willd. seeds. Bot. Bull. Acad. Sin. 41: 27-34.

Qin, C., C. Yu, Y. Shen, X. Fang, L. Chen, J. Min, J. Cheng, S. Zhao, M. Xu, Y. Luo, Y. Yang, Z. Wu, L. Mao, H. Wu, C. Ling-Hu, H. Zhou, H. Lin, S. González-Morales, D. L. Trejo-Saavedra, H. Tian, X. Tang, M. Zhao, Z. Huang, A. Zhou, X. Yao, J. Cui, W. Li, Z. Chen, Y. Feng, Y. Niu, S. Bi, X. Yang, W. Li, H. Cai, X. Luo, S.

Montes-Hernández, M. A. Leyva-González, Z. Xiong, X. He, L. Bai, S. Tan, X. Tang, D. Liu, J. Liu, S. Zhang, M. Chen, L. Zhang, L. Zhang, Y. Zhang, W. Liao, Y. Zhang, M. Wang, X. Lv, B. Wen, H. Liu, H. Luan, Y. Zhang, S. Yang, X. Wang, J. Xu, X. Li, S. Li, J. Wang, A. Palloix, P. W. Bosland, Y. Li, A. Krogh, R. F. Rivera-Bustamante, L. Herrera-Estrella, Y. Yin, J. Yu, K. Hu and Z. Zhang. 2014. Whole-genome sequencing of cultivated and wild peppers provides insights into *Capsicum*. Domestication and specialization. Proc. Natl. Acad. Sci. USA 111: 5135-5140.

Rafael, U. L., R. I. D. de la Garza and I. Juan. 2014. Effects of substrate salinity and nutrient levels on physiological response, yield, and fruit quality of Habanero pepper. Mexico Hort. Sci. 49: 812-818.

Rathnayaka, R. M. S. M. B., M. Minami, K. Nemoto, S. P. Sudasinghe and K. Matsushima. 2021. Relationship between water supply and sugar and capsaicinoids content in fruit of chili pepper *(Capsicum annuum* L.*)*. J. Hort. Sci. (Japan) 90: 58-67.

Rathnayaka, R. M. S. M. B., S. P. Sudasinghe, M. Minami, K. Nemoto and K. Matsushima. 2020. Effect of salinity stress in soil on the taste component in Chili Pepper (*Capsicum* spp.). Hort. Sci. 19 (Extra issue 1): 157.

Rowland, B. J., B. Villalon and E. E. Burns. 1983. Capsaicin production in Sweet Bell and pungent Jalapeno peppers. J. Agric. Food Chem. 31: 484-487.

Ruiz-Lau, N., F. M. Lara, Y. M. Garcia, E. Z. Moreno, A. G. Antonio, I. E. Machado and M. M. Estevez. 2011. Water deficit affects the accumulation of capsaicinoids in fruits of *Capsicusm chinense* Jacq. Hort. Sci. 46: 487-492.

Sadamasa, T. 2008. Influence of growing environments on chemical and mineral constituents of tomato leaves and taste constituents of tomato fruits. Fukui Agri. Ex. Station. 45:35-42.

Saito, T., C. Matsukura, Y. Ban, K. Shoji, M. Sugiyama, N. Fukuda and S. Nishimura. 2008. Salinity stress affects assimilate metabolism at the gene-expression level during fruit development and improves fruit quality in tomato (*Solanum lycopersicum* L.). J. Japan. Soc. Hort. Sci. 77: 61-68.

Saito, T., N. Fukuda and S. Nishimura. 2006. Effects of salinity treatment duration and planting density on size and sugar content of hydroponically grown tomato fruits. J. Japan. Soc. Hort. Sci. 75: 392-398.

Sakamoto, Y., S. Watanabe, T. Nakashima and K. Okano. 1999. Effects of salinity at two ripening stages on the fruit quality of single-truss tomato grown in hydroponics. J. Hort. Sci. Biotech. 74: 690-693.

Shahbaz, M. and M. Ashraf. 2013. Improving salinity tolerance in cereals. Crit. Rev. Pl. Sci. 32: 237-249.

Shrivastava, P. and R. Kumar. 2015. Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi J. Biol. Sci. 22: 123-131.

Siringam, K., N. Juntawong, S. Cha-um and C. Kirdmanee. 2011. Salt stress induced ion accumulation, ion homeostasis, membrane injury and sugar contents in salt-sensitive rice (*Oryza sativa* L. spp. *indica)* roots under isosmotic conditions. Afr. J. Biotechnol. 10: 1340-1346.

Stewart, C., B. C. Kang, K. Liu, M. Mazourek, S. L. Moore, E. Y. Yoo, B. D. Kim, I. Paran and M. M. Jahn. 2005. The *Pun1* gene for pungency in pepper encodes a putative acyltransferase. Plant J. 42: 675-688.

Stewart, C., M. Mazourek, G. M. Stellari, M. O'Connell and M. Jahn. 2007. Genetic control of pungency in *C. chinense* via the *Pun1* locus. J. Exp. Bot. 58: 979-991.

Sugiyama, R., M. Shite, H. Fujino, Y. Tatsuo, S. Nakamura, N. Kakusho, M. Ito, H. Yokota, K. Kase and F. Kurosaki. 2006. Estimation of capsaicinoid biosynthetic capacity from capsaicinoid content and surface area of the placental dissepiment in Capsicum fruits. Plant Morphology. 18:75-82. (In Japanese with English summary).

Sung, Y., Y. Y. Chang and N. L. Ting. 2005. Capsaicin biosynthesis in water-stressed hot pepper fruits. Bot. Bull. Acad. Sin. 46: 35-42.

Suzuki, T. and K. Iwai. 1984. Constituents of red pepper species: Chemistry, biochemistry, pharmacology, and food science of the pungent principle of *Capsicum* species. The alkaloids: Chemistry and pharmacology. 23: 227-299.

Tadesse, T., E. W. Hewett, M. A. Nichols and K. J. Fisher. 2002. Changes in physicochemical attributes of sweet pepper cv. Domino during fruit growth and development. Hort. Sci. 93: 91-103.

Tanaka, Y., F. Nakashima, E. Kirii, T. Goto, Y. Yoshida and K. Yasuba. 2017. Difference in capsaicinoid biosynthesis gene expression in the pericarp reveals elevation of capsaicinoid contents in chili peppers (*Capsicum chinense*). Plant Cell Rep. 36: 267-279.

Tanaka, Y., M. Hosokawa, T. Miwa, T. Watanabe and S. Yazawa. 2010. Novel loss-of-function putative aminotransferase alleles cause biosynthesis of capsinoids, nonpungent capsaicinoid analogues, in mildly pungent chili peppers (*Capsicum chinense*). J. Agric. Food Chem. 58: 11762-11767.

Tanaka, Y., T. Asano, Y. Kanemitsu, T. Goto, Y. Yoshida, K. Yasuba, Y. Misawa, S. Nakatani and K. Kobata. 2019. Positional differences of intronic transposons in *pAMT* affect the pungency level in chili pepper through altered splicing efficiency. Plant J. DOI: 10.1111/tpj. 14462.

Ullah, S. M., G. Soja and M. H. Gerzabek. 1993. Ion uptake, osmoregulation and plant water relations in faba bean (*Vicia faba* L.) under salt stress, Die Bodenkultur. 44: 291-301.

Ullah, S. M., M. H. Gerzabek and G. Soja. 1994. Effect of sea water and soil salinity on Ion uptake, yield and quality of tomato (fruits). Die Bodenkultur. 45: 227-237.

UNESCO Water Portal 2005. [Online] <http://www.unesco.org/water/news/newsletter/117.shtml> (browsed on May 12, 2020).

Ungar, I. A. 1991. Ecophysiology of Vascular Halophytes. Cambridge University, CRC Press. Boca Raton, FL, USA. [Online] <https://doi.org/10.1017/S0014479700021335> (browsed on March 15, 2020).

Van der Beek, J. G. and A. Ltifi. 1991. Evidence for salt tolerance in pepper varieties (*Capsicum annuum* L.) in Tunisia. Euphytica 57: 51-5.

Wu, M. and C. Kubota. 2008. Effects of high electrical conductivity of nutrient solution and its application timing on lycopene, chlorophyll and sugar concentrations of hydroponic tomatoes during ripening. Hort. Sci. 116: 122-129.

Yamaguchi, T. and E. Blumwald. 2005. Developing salt-tolerant crop plants: challenges and opportunities. Trends Pl. Sci. 10: 615-620.

Yin, Y. G., Y. Kobayashi, A. Sanuki, S. Kondo, N. Fukuda, H. Ezura, S. Sugaya and C. Matsukura. 2010. Salinity induces carbohydrate accumulation and sugar-regulated starch biosynthetic genes in tomato (*Solanum lycopersicum* L. cv. 'Micro-Tom') fruits in an ABA- and osmotic stress-independent manner. J. Exp. Bot. 61: 563-574.

Zewdie, Y. and P. W. Bosland. 2000. Evaluation of genotype, environment, and genotype- by environment interaction for capsaicinoids in *Capsicum annuum* L. Euphytica. 111: 185-190.

Zhani, K., H. Nina, A. Rezwan and H. Cherif. 2012. Evaluation of salt tolerance (NaCl) in Tunisian chili pepper (*Capsicum frutescens* L.) on growth, mineral analysis and solutes synthesis. J. Stress Physiology & Biochemistry 9: 209-228.

Zhu, Z., B. Sun, W. Cai, X. Zhou, Y. Mao, C. Chen, J. Wei, B. Cao, C. Chen, G. Chen and J. Lei. 2019. Natural variations in the MYB transcription factor MYB31 determine the evolution of extremely pungent peppers. New Phytol. 223: 922-938.