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Effect of water stress and aphid herbivory on flavonoids in broccoli (*Brassica oleracea* var. *italica* Plenck)

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Summary

The aim of this study was to determine changes within flavonoid levels in broccoli plants (*Brassica oleracea* var. *italica* Plenck) induced by water stress conditions and aphid herbivory. Three water treatments, well-watered, drought, and water-logged, were applied to plants. Two aphid species, the specialist *Brevicoryne brassicae* (L.) and the generalist *Myzus persicae* (Sulzer), were used to evaluate combination effects under water stress. Drought and water-logged stress decreased plants water content and reduced the growth of broccoli. Analysis of flavonoids revealed that the broccoli variety 'Calabrese' only contained kaempferol. The kaempferol content was similar in all experimental plants after one week cultivation under various water treatments. However, after two weeks of water stress, significantly higher kaempferol amounts were detected in drought and water-logged broccoli plants compared to well-watered plants. Herbivory of the aphids' *B. brassicae* and *M. persicae* did not change the level of kaempferol in broccoli, regardless of the water treatment.

Introduction

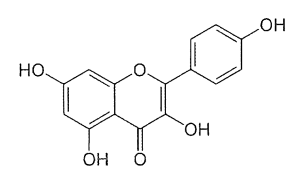
The *Brassica* genus includes some of the most popular vegetables consumed all over the world and is considered to be a good source for bioactive phytochemicals. Within this family, broccoli, *Brassica oleracea* var. *italica* Plenck, is cultivated worldwide in the temperate and subtropical region. The chemical composition of broccoli makes it popular for the human diet since it contains secondary metabolites such as glucosinolates and flavonoids, some of them showing health promoting effects (PODSĘDEK, 2007). These secondary metabolites also play a key role in insect-plant interactions and plant resistance (HOPKINS et al., 2009; SIMMONDS, 2003).

The antioxidant activity of flavonoids is based on their hydroxylated ring structure (Fig. 1) and supposed to protect humans against several types of cancer (KNEKT et al., 2002) and cardiovascular diseases (CHU et al., 2000). Kaempferol, a predominated flavonoid found in *Brassica* crops (HUANG et al., 2007; HEIMLER et al., 2006) has strong antioxidant potential (KIM et al., 2006) and higher intakes resulted in lower risk of heart diseases (LIN et al., 2007). Recent studies have demonstrated that quercetin and kaempferol suppress cell proliferation in human gut cancer lines (ACKLAND et al., 2005). Due to some health promoting activities of flavonoids, in the recent year's plant scientists have been working with different crop species, cultivars, agronomic practices, and post harvest operations to ensure higher flavonoid content in crops (SCHMIDT et al., 2010, PODSĘDEK, 2007; HAGEN et al., 2009; STEWART et al., 2001). Additionally, flavonoids are important plant defence compounds (SIMMONDS, 2003; ORR and SOHAL, 1994). The flavonoid quercetin in particular can stimulate insect feeding (RUUHOLA et al., 2001), whereas isoflavonoids can have negative effects on herbivores (SIMMONDS and STEVENSON, 2001; YU et al., 2003). Thus the role of flavonoids in insect-plant interactions appears to be complex.

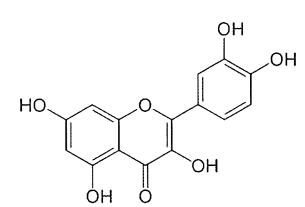
The concentration of flavonoids in plants depends on cultivars, plant age, and soil nutrition status. But also climatic conditions, such as temperature, concentration of CO_2, radiation and biotic factors, such as insect herbivory and pathogen attack, are influencing flavonoid levels (O'NEILL et al., 2010; SCHMIDT et al., 2010; STEWART et al., 2001; HOFMANN et al., 2000; TREUTTER, 2006). Plants exposed to UV light, elevated CO_2 levels, and insect herbivory have been shown to accumulate higher flavonoid contents.

Water stress is an important abiotic stress factor in plant production systems in many parts of the world. Under drought and water logging conditions chemical compositions of plants is altered. Both, the primary and secondary metabolites in broccoli, are affected by water stress (KHAN et al., 2010). Broccoli contains high amounts of flavonoids (PODSĘDEK, 2007). It is most likely, that water stress condition and insect feeding alters the flavonoid content and/or profile in broccoli.

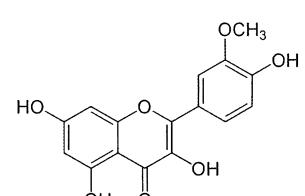
The cabbage aphid, *Brevicoryne brassicae* (L.) and the peach potato aphid, *Myzus persicae* (Sulzer), are two important pests of broccoli in Europe and North-America. Feeding of these two aphids changes the profile of glucosinolate in broccoli (KHAN et al., 2011) and might also affect flavonoid contents. Considering all, the present study was undertaken to determine changes within flavonoids in broccoli induced by water stress and aphid feeding.



Kaempferol



Quercetin



Isorhamnetin

Fig. 1: Structures of flavonoids commonly found in *Brassica* crops.

Materials and methods

Host plant

Broccoli plants were used to assay the effect of various plant water statuses and aphid feeding on flavonoid contents. Plants were grown in a green house at a temperature of 22 ± 1°C and photoperiod with 16-h light (200 µmol m^{-2} s^{-1} light intensity). Seeds were sown in trays filled with potting substrate (Gramoflor Profi-Substrat, Nutrient available/l: 50 - 300 mg N, 80 - 300 mg P_2O_5, 80 - 400 mg K_2O) and one week old seedlings were transferred to pots (one seedling per pot, pot size: 10 x 10 cm). All plants were watered as needed to maintain regular growth.

Aphids rearing

Brevicoryne brassicae (L.) and *Myzus persicae* (Sulzer) were collected from different cabbage fields in the federal states of Berlin and Brandenburg, Germany, and were reared on *Brassica rapa* ssp. *chinensis* (pak-choi) plants in a climate room with 22±1°C temperature and supplemental light for 14 hours.

Water stress treatment

Different water regimes were applied to achieve drought, well-watered, and water-logged conditions in the soil and which allowed plant growth. Each broccoli plant received 600, 200, and 50 ml water/week in water-logged, well-watered, and drought treatments, respectively. Water stress treatments were started when plants were 14 days old and water was delivered four times per week. Pots were placed in 4 cm deep plastic dishes to retain any water.

Experimental procedure

After one week of different water stress treatments, ten plants per treatment were harvested in pairs by cutting at the base, flash frozen in liquid nitrogen and stored at -80°C. On the same day, twenty *B. brassicae* adults per plant or twenty *M. persicae* adults per plant were released on the plants, by using a fine brush. Afterwards, each plant was caged individually in special transparent plastic cylinders (cages) covered with gauze (mesh size 0.1 mm × 0.1 mm) to prevent aphids escaping. The aphid free control plants from each water treatment were also caged. The experiment was done in ten replications per treatment. After one week, plants were harvested in pairs and combined, thus five replicates per treatment were used for analysis of flavonoids. Control plants were cut directly at the base and immediately flash-frozen in liquid nitrogen. In aphid treatments, the aphids were removed with a brush and following the plants were frozen in liquid nitrogen. All materials were stored at -80°C until they were freeze dried and grounded for chemical analysis. A parallel experiment was conducted with ten replicates for each treatment to measure the water content of soil and plants. Water content was measured by gravimetric method, where the fresh weight of soil and plants were recorded and later on the dry weight were determined after 48 h drying at 105°C in an oven. The following formula was used: *Water content (%) = (fresh weight – dry weight)/dry weight ×100.*

Chemical analysis of flavonoids

For the extraction of flavonoid compounds, 20 mg grounded sample was dissolved in 750 µl 80% methanol (pH = 4) and sonicated at 4°C temperature for 20 minutes. After 5 min centrifugation at 4500 rpm, the extract was collected and the pellet was re-extracted twice more. Methanol from the combined extract was removed to near dryness in a centrifugation evaporator (Speed Vac, SC 110) at room temp. Equal volume of 2M HCl (250 µl) was added to the extract and heated at 90°C for 2 hours in a thermo mixer at 400 rpm (Eppendorf, Germany) to form aglycones of flavonoids. The extract was cooled down and 100% methanol (HPLC grade) was added up to 1 ml final volume. The extract was then filtered through Spin-X Centrifuge Tube Filters (Sigma-Aldrich, Inc.) and the flow through was collected in 2 ml HPLC vials.

Extracts were run on a Dionex Summit P680A HPLC system, equipped with an ASI-100 auto sampler and a PDA-100 photodiode array detector. The aglycones of flavonoids were separated on a narrow bore 3 µm column (AcclaimPA C16, 2.1 × 150mm, Dionex) at a flow rate of 0.4 ml/min and a column temperature of 25°C. A 32 min gradient program was used with eluents Milli-Q water (pH 3 with acetic acid) and 100% acetonitrile (HPLC grade). Flavonoids aglycone peaks were monitored at 370 nm. Flavonoids identification was performed using standard, retention time, and UV spectra. The flavonoids quantity was calculated from HPLC peak areas using calibration curve from flavonoids aglycone standards at 370 nm.

Statistics

Analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test was used to calculate significant differences between treatments. All tests were performed at a significance level of $P < 0.05$ using the statistical program SPSS (version 16).

Results

Water content of soil

There was a significant difference in water content of soils receiving different amounts of water in the treatments (one way ANOVA: $F_{2, 29} = 228$, $P = 0.000$ for 1 week; $F_{2, 29} = 224$, $P = 0.000$ for 2 weeks) (Fig. 2). The water content in water-logged soil was 79% and 80%, in well-watered soils 73% and 68%, and in drought stressed soils 39% and 38% after one week and after two weeks of treatment, respectively.

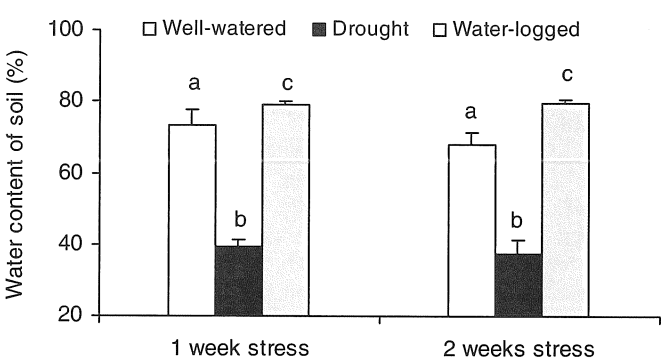


Fig. 2: Water contents of soils in the different water treatments (plants 21 days old after 1 week stress and 28 days old after two weeks, different small letters indicate significant differences between water treatments within stress duration, Tukey's HSD Test, $P < 0.05$).

Water content and growth of plants

The water content of broccoli plants greatly depended on the status of soil water. Significant differences were found between treatments (one way ANOVA: $F_{2, 29} = 6$, $P = 0.016$ for 1 week; $F_{2, 29} = 15$, $P = 0.001$ for 2 weeks of stress), and highest water content was measured in well-watered plants followed by water-logged and drought stressed plants (Fig. 3). Growth of broccoli plants depended on water stress conditions and significant differences were found in dry weights among treatments (one way ANOVA, $F_{2, 14} = 8$, $P = 0.007$ for

1 week, $F_{2, 14} = 11$, $P = 0.002$ for 2 weeks of stress). Highest growth was found in those broccoli plants which were grown under well-watered condition. Both drought and water-logged conditions resulted in decreased plants growth (Fig. 3).

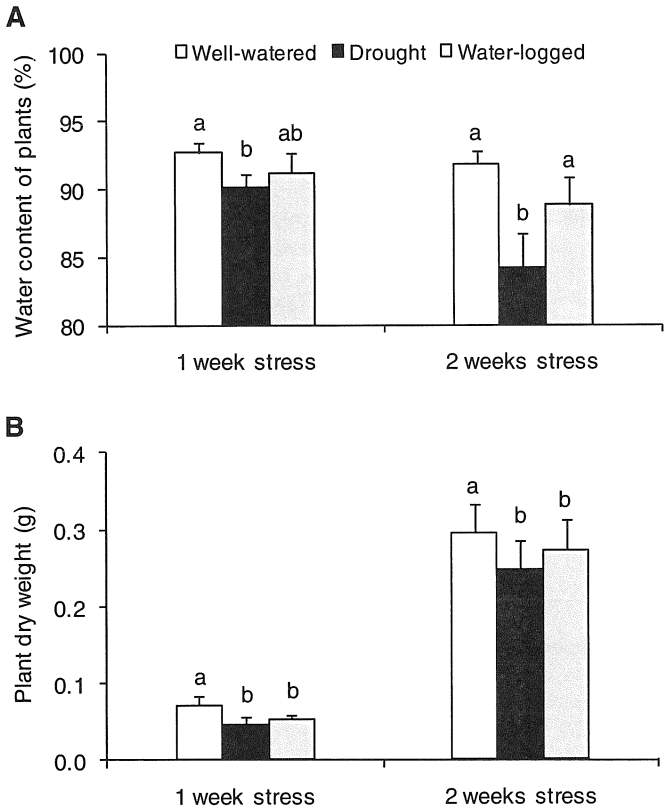


Fig. 3: Effect of water stress on (A) water content and (B) dry weight of broccoli plants (different small letters capping bar indicate significant differences between treatments within time, Tukey's HSD Test, $P < 0.05$).

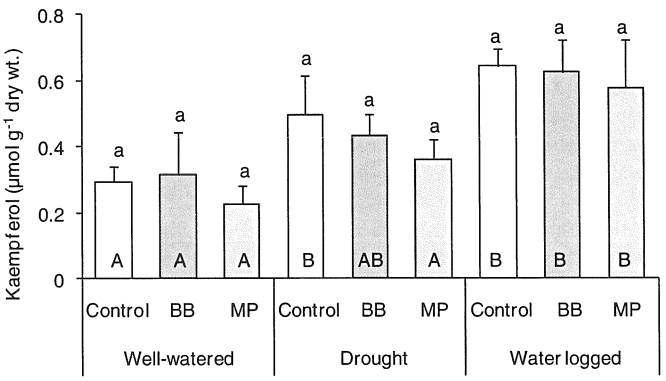


Fig. 4: Kaempferol content after 2 weeks of water stress in control and aphid treated broccoli plants (different small letters indicate significant differences within water treatments; different capital letters indicate significant difference between water treatments for control and aphid treatments, Tukey's HSD Test, $P < 0.05$; BB indicate *B. brassicae*, MP indicate *M. persicae*).

Flavonoids content

Analysis of flavonoids using HPLC revealed that 21 and 28 days old broccoli plants (*Brassica oleracea* var. *italica* Plenck) 'Calabrese' contained kaempferol as main flavonoid. The level of kaempferol did not change significantly after one week exposure to different water regimes (one way ANOVA, $F_{2,14} = 0.6$, $P = 0.560$). However, after two weeks cultivation under different water conditions, a significantly higher kaempferol content was detected in water-logged and drought stressed plants compared to well-watered plants (one way ANOVA, $F_{2,14} = 21$, $P = 0.000$; Fig. 4). *B. brassicae* and *M. persicae* feeding did not changed kaempferol levels in broccoli plants within each treatment (one way ANOVA: $F_{2,14} = 1$, $P = 0.325$ for well-watered treatment, $F_{2,14} = 2$, $P = 0.138$ for drought and $F_{2,14} = 0.4$, $P = 0.677$ for water-logged treatments; Fig. 4). However, the induced kaempferol levels after feeding of both aphids differed between water treatments (one way ANOVA: $F_{2,14} = 13$, $P = 0.001$ for *M. persicae*, $F_{2,14} = 9$, $P = 0.005$ for *B. Brassicae*), while higher kaempferol contents were found in water-logged plants followed by drought and well-watered plants (Fig. 4).

Discussion

Water stress induced changes in flavonoids of broccoli was not investigated previously. However, other crops were used to study water stress induced changes within flavonoids, for example rice, sweet potato, and citrus plants (Lin et al., 2006; Chutipaijit et al., 2008; Hernández et al., 2004). In broccoli, the levels of kaempferol remained unchanged up to one week cultivation under different water conditions. After two weeks of water stress significant changes were found in kaempferol when compared to plants grown under well-watered conditions. Here, drought and water-logged plants contained higher kaempferol levels than well-watered plants. These findings indicate that the plant response towards water stress with changing kaempferol contents is, compared to other reactions such as the increase of osmo-protectants, relatively slow (Khan et al., 2010). Furthermore, our study showed that aphid feeding had no effect on kaempferol levels in broccoli plants grown at various water statuses. Similarly, in soybean (*Glycine max* L.) aphid feeding did not change kaempferol levels (O'Neill et al., 2010.

Stressed plants, grown under drought as well as water-logged conditions, showed a decreased biomass production. On the other hand kaempferol content was higher in drought and water-logged plants, suggesting that plants produced kaempferol as biochemical adaptation towards water stress. Decreased plant growth might be related to higher kaempferol content as plants need to invest photosynthates as resources of carbon required to build the C6-C3-C6 flavone skeleton for the kaempferol biosynthesis. Recently, in a model calculation, Gayler et al. (2004) showed that defensive flavonoids are expensive and their accumulation may take place at the expense of plant's growth. Furthermore, there are numerous reports about the increase of flavonoids, where simultaneously, growth is reduced (Stewart et al., 2001; Hofmann et al., 2000).

Drought stress leads to an increase of reactive oxygen species (ROS) which can inactivate various Calvin-cycle enzymes and are involved in oxidative damage in plants (Chaudiere and Ilious, 1999; Schwanz et al., 1996; Carvalho and Amancio, 2002; Keles and Dunl, 2002; Sairam et al., 1997). The ROSs can be removed both enzymatically and chemically to protect plant cells against oxygen toxicity and counter the hazardous effects of ROSs under stress (Perata and Alpi, 1993). Major non-enzymatic antioxidants including ascorbate, tocopherol, alkaloids, glutathione, flavonoids, and anthocyanin in plants have been reported (Mittler, 2002). The flavonoids components are known to be powerful hydrogen-donating antioxidants and scavengers of ROSs and are accumulated in responses to UV-B, cold, salt, and drought as reported earlier by Chalker-Scott (1999) and Zietz et al. (2010). Higher kaempferol

content in drought and water logged broccoli plants might be an adaptation of plants to combat the activity of ROSs and prevent further plant damage.

This study clearly shows that there are significant effects of water supply on the concentration of bioactive flavonoids in broccoli plants. But on the other hand at the same time other secondary metabolites such as glucosinolates can decrease under drought stress increasing the risk of herbivory (KHAN et al., 2010). Also by reducing water supply or providing excess water the yield is affected and therefore, further studies are needed before giving recommendations for water application strategies to improve product quality of broccoli.

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