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Time set of inflorescence emergence stage independent from delayed ontogenesis by drought periods in winter rape

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ABSTRACT

Drought stress affects plant physiology and phenology and therefore influences crop productivity. The impact of different periods of water stress on various physiological parameters in winter rape (*Brassica napus*) was studied under climate chamber conditions. Drought periods applied to various ontogenetic stages of winter rape plants resulted in water loss, photosynthesis decrease, delayed ontogenesis as well as loss of biomass and yield. Furthermore, vacuolar, cytosolic and extracellular invertases were strongly downregulated on the 29th day after vernalization (d.a.v.), which was also the case in control plants, concurrently in the phase of inflorescence development. This time effect was also found by means of corrected osmolality. Unaffected by stress application, the corrected osmolality decreased significantly on the 29th d.a.v.. Between the 35th and the 45th d.a.v., corrected osmolality increased markedly in all variants, as control plants matured from main inflorescence to flowering stage. In the following stage of fruit development, stressed plants differed significantly from control regarding corrected osmolality and invertase activities indicating stress compensation reactions. However, the duration of the stress compensation reaction was found to be limited by fixed time signals. Stress tolerance thus seems to be related to a more flexible reaction to fixed time signals in order to fully use the existing compensatory potential.

KEYWORDS

Drought;
Rape;
Ontogenesis;
Invertase;
RWC;
Chlorophyll.

INTRODUCTION

Drought, in conjunction with coincident high temperature, is the most important environmental constraint to plant survival and crop productivity[1]. Typical stress responses involve accumulation of osmolytes, amino acids and modulation of sugar metabolism and loss of photosynthetic activity[2]. Photosynthesis is one of the first processes affected in plants. Thus the following depletion of energy and sugars imposes a stressful metabolic situation[3]. Consequently, inhibition of plant growth results in losses of crop yield. However, the timing of water deficits during the season may have a much larger impact on yield than the intensity of drought[4].

The major plant response at the cellular level in adaptation to drought, which has a proven effect on yield under drought stress, is osmotic adjustment[5], (for review see[6]). Another plant response to drought is the change in carbohydrate metabolism, which may be directly related to dehydration tolerance[7]. Cuellar-Ortiz et al.[8] showed that carbohydrate partitioning is affected by drought in common beans. They found that the modulation of carbohydrate partitioning towards seed filling is a successful strategy in the development of drought resistant cultivars. Hexoses and sucrose are not only substrates for metabolism, but also play a regulatory role in germination and seedling development in the complex sugar–hormone interaction manner[9]. Bogdan and Zagdańska[10] report that the dehydration tolerance level in wheat seedlings is regulated by the sucrose catabolism and an invertase/sucrose synthase balance. Luquet et al.[11] investigated the carbohydrate dynamics and source-sink relationships among organs in young rice plants in the course of dry-down cycles. In source leaves they found an increased hexose concentration and a decrease of starch content, and *vice versa* in sink leaves and roots. Furthermore, the authors observed that the invertase gene expression is mostly upregulated under stress in young leaves (sink) and downregulated in mature leaves (source). Stress induced gene expression is closely correlated to normal plant growth and development, i.e. it modulates sink source relationships[12].

Oilseed rape (*Brassica napus* L.) is one of the most important edible oilseed crops in the world, grown for oil production and protein in animal feed. Rape is especially susceptible to water stress during the flowering period and during pod development[13]. However, we recently showed that oilseed rape is also vulnerable in the shooting stage[14]. Although the ontogenetic stages of stressed and unstressed plants are the same at the time of harvest, the development of seed biomass is significantly depressed under stress. Individual plant responses to drought stress are indicated by the activity of the extracellular invertase and the osmolality in leaves. Based on the preceding study, the following topics were addressed: (i) source-sink regulations in carbohydrate partitioning indicated through invertase activities and (ii) seed biomass development under drought stress and its recovery in various ontogenetic stages as well as (iii) osmolytes induced in stressed oilseed rape. Periods of drought were applied at the stage of shooting, flowering and over both stages.

METHODS

Seeds of *Brassica napus* (cultivar Titan) were sown in containers (diameter 20 cm x 20 cm height) filled with 8 kg ready-made soil (type ED73, Einheitserdewerk Uetersen W. Tantau GmbH & Co. KG, Germany) and grown in a climatic chamber (KTLK 2000, Nema, Netzschkau/ Vötsch, Germany) for 5 weeks. The vernalization stage occurred at 1°C for 4 weeks with a light/dark rhythm ratio of 8 hours/16 hours and light intensity of 595 µmol m^{-2} s^{-1} (end of vernalization). Thereafter, the temperature was increased stepwise every two days by 4 degrees up to 15°C / 9°C with light periods of 10 hours. For the following 80 days, the light was set to 16 hours for the remaining 26 days pod filling occurred at a set temperature of 20°C/12°C.

The initial content of water in the ready-made soil was 66%. A group of 68 plants (control) was watered daily with deionized water to 85-90% of the water holding capacity of the soil. The water amount was adjusted according to each container weight and consumed water was supplemented. The content of soil water was determined every two days so that plant biomass increase could be accounted for. The drought stress treatments were: (i) stress imposed at early vegetative stage (S_E); (ii) stress imposed at inflorescence emergence (S_I); (iii); prolonged drought/stress applied to groups of plants (S_P) = (S_E+ S_I) (TABLE 1).

TABLE 1 : Number of plants (per variant and harvest).

	Total number of plants	regular harvest (BBCH>78)	interim harvest (BBCH<78)
control	68	22	6x6 plants +1x10 plants
S_P	38	14	4x6 plants
S_E	50	14	6x6 plants
S_I	44	14	5x6 plants
summa	200	64	136

All measurements were performed on the fourth leaf from the top of the plant, as this was found to be the first fully developed leaf (in terms of size) for the

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various growth stages (TABLE 2).

TABLE 2 : Description of ontogeny for the various drought stress treatments: Plant development (BBCH); days after vernalization (d.a.v.); S_E - stress imposed at early vegetative stage for 15d; S_I - stress imposed at inflorescence emergence for 15 d; S_P - prolonged drought/stress = (S_E+ S_I) for 30 d.

	d.a.v.	BBCH			
		co	S_E	S_p	S_I
	0	15			
	17	16			
start of S_E and S_P	18				
	22	16	16	16	
	26	18	16	16	
	28	50	16	16	
	31	51	17	16	
end of S_E, start of S_I	33				
	35	55	17	16	55
	38	63	52	15	62
	42	67	56	15	65
	45	67	60	15	65
end of S_P and S_I	48				
	50	72	66	17	68
	53	74	66	50	70
	56	78	70	51	70
	66	77	73	54	70
	73	76	72	61	72
	82	84		68	72
	102	88	86	75	83
	112		87	78	87

Three discs of 6 mm diameter were cut out of the leaf for measurements of osmolality, relative water content (RWC) and dry matter using a laboratory cork borer. Immediately thereafter, the leaves were removed from the plants and conserved at –80°C for enzyme analyses. Measurements of plant physiologic parameters were logged up to the 71st d.a.v..

The biomass was determined as follows: Fresh matter of plant shoots and separated pods was weighed immediately after removal, and after drying at 50°C for 48 hours for dry matter determination.

Chlorophyll fluorescence, photosynthesis and transpiration

A portable chlorophyll fluorometer PAM 2100 (Walz, Effeltrich, Germany) was used to measure the maximum efficiency of photosystem II[15]. The Genty-parameter Fv/Fm comes from the equation

Fv/Fm = (Fm-Fo) / Fm,

(Fo is the fluorescence in the absence of actinic (photosynthetic) light, and Fm is the maximum fluorescence following a highly intensive, short flash of light). The chlorophyll fluorescence was determined during the dark phase of the diurnal cycle under green light inside the growth chamber. For this purpose, consecutive measurements were conducted at 4 different sites of the leaf. Two hours after the end of the dark phase, the net photosynthesis and transpiration rates were measured on the third leaf from top of the plant using the HCM 1000 (Walz, Effeltrich). Temperature and PPFD were kept constant at 20°C and 400 µmol m^{-2} s^{-1}, respectively.

Osmolality

The osmolality was measured with 10 µL of supernatant and a vapor pressure osmometer (Vapro 5520, Wescor, Inc., USA; described by[14]). Measurements were performed in triple replicates. The results were expressed in mmol of all solutes present in one kg of water in the plant sap. The values have to be corrected by means of a multiplication with the relative water content (RWC), further referred to as corrected osmolality.

Relative water content (RWC)

RWC of leaf tissue was calculated according to Barr and Weatherley[16] in leaf discs (described by Müller et al.[14]).

Invertase activity analysis

The homogenization of plant material and the enzyme activity analyses were carried out as described by[14].

Statistics

All calculations were performed with the adequate analytic module of the Statistica software (StatSoft, Inc., Tulsa, OK/USA, version 7.1., http://www.statsoft.com). Differences in the means of the fresh and dry matter biomass were tested for statistical significance by using the t-test Scheffé. Data from the different variables and times were subjected to a multifactorial ANOVA, and significances were tested post hoc using the Fisher-LSD-test ($P < 0.05$). Interactions and correlations between

biochemical or physiological data and dry matter biomass were ascertained by means of Spearman rank correlation coefficient analysis.

RESULTS

Periods of no watering applied during various ontogenetic stages induced similar plant reactions. Osmotic pressure in leaves was significantly increased, but corrected osmolality was not significantly different from control up to the 45th day after vernalisation (d.a.v.) (Figure 1).

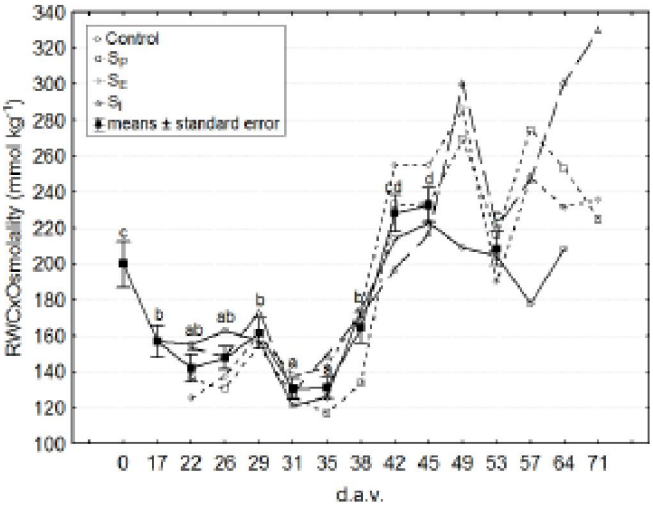

Figure 1 : Corrected osmolality influenced by drought stress (S_E = early stress, S_P = prolonged stress, S_I = inflorescence stress), mean was calculated where no significant differences between variants were detected (letters mark significant differences between means).

Increased osmotic pressure was caused only by water loss, not by an extra production of osmotic substances. In all variants corrected osmolality was increased significantly till 29th d.a.v. where control plants reached BBCH 50, but stress plants only BBCH 16 (Figure 2). Decreased corrected osmolality at 31st and 35th d.a.v. followed by an increase up to 42nd/ 45th d.a.v. was detected in all plants without significant differences between variants. At the 42nd/ 45th d.a.v., control plants were in transition from flower to fruit development; in contrast plants under prolonged stress application remained in vegetative stage (BBCH 17).

On the 49th d.a.v. corrected osmolality of control plants differed significantly from all stress variants. At this point control plants were in the stage of further fruit

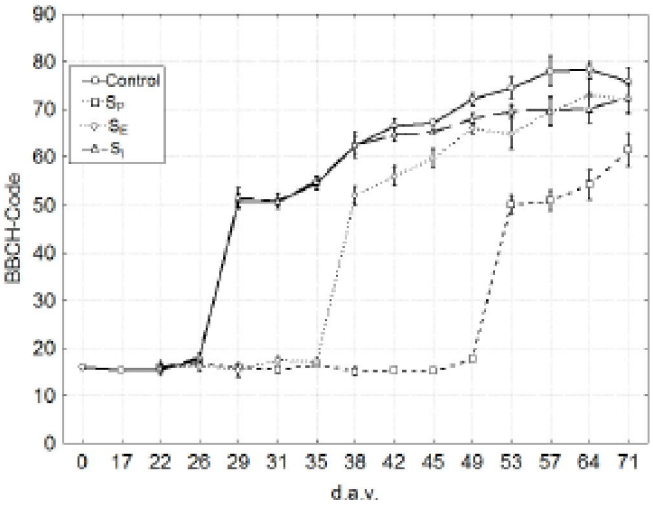

Figure 2 : Plant development (BBCH-code) influenced by drought stress (S_E = early stress, S_P = prolonged stress, S_I = inflorescence stress).

development (BBCH 72) while stress variants were ontogenetically delayed: S_E at BBCH 66, S_I at BBCH 68 and S_P at BBCH 17. On the 53rd d.a.v. corrected osmolality of all variants matched. In the control variant, 40% of pods have reached final size. Following the 53rd d.a.v, corrected osmolality significantly increased to different levels in all stress plants.

Invertase activities were not significantly different to control up to the 31st d.a.v. and showed the same dynamics as corrected osmolality, with a pronounced peak at 29th d.a.v. followed by a decrease up to 31st d.a.v. (Figure 3). Cytosolic and vacuolic invertase activities of control and stressed plants matched at 45th d.a.v., extracellular invertase activities at 49th d.a.v. (Figure 4).

Between these points in time, which were independent of stress impact and ontogenetic stage, stress and recovery reactions were observed. Corrected osmolality showed no specific stress reaction of plants. Following the end of stress impacts (S_E = 33rd day, S_P and S_I = 48th day) all stress variants had a significantly enhanced level of corrected osmolality on the 49th and 57th d.a.v..

Cytosolic and vacuolic invertase activities were nearly equal and showed stress specific reactions. Prolonged stress resulted in an activity similar to that found in control plants between 35th and 45th d.a.v.. After stress impact (48th d.a.v.), activity reached a 4fold higher level than control on the 53rd d.a.v.. Furthermore, plants rapidly grew flower buds (BBCH 50 on the 53rd d.a.v.).

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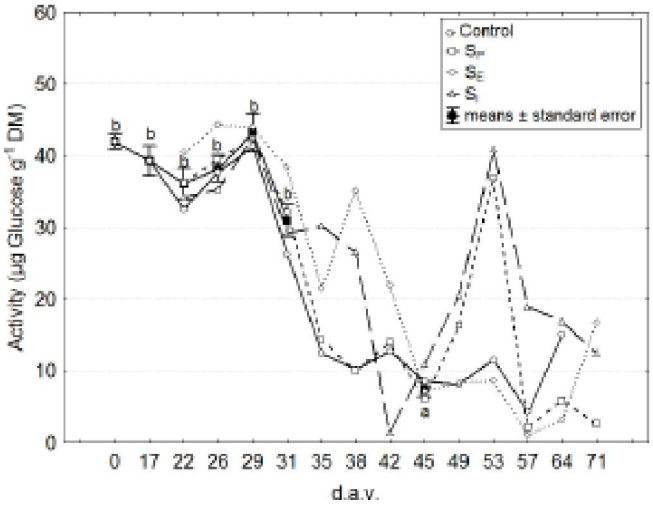

Figure 3 : Cytosolic invertase activity influenced by drought stress (S_E = early stress, S_P = prolonged stress, S_I = inflorescence stress), mean was calculated where no significant differences between variants was detected, significant differences are marked by letters.

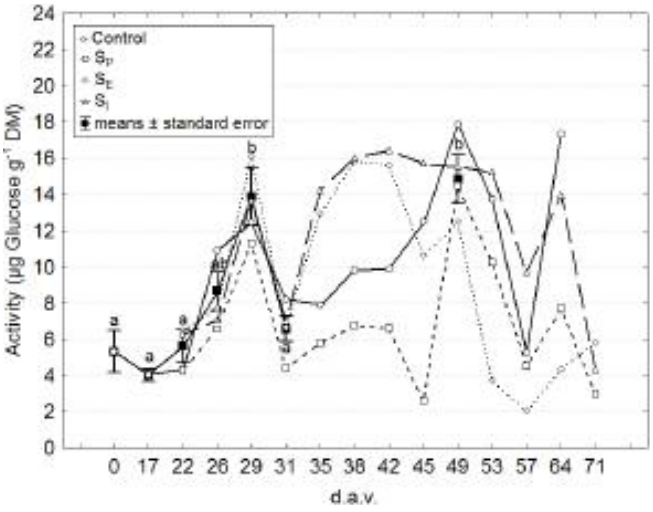

Figure 4 : Extracellulare invertase activity influenced by drought stress (S_E = early stress, S_P = prolonged stress, S_I = inflorescence stress), mean was calculated where no significant differences between variants were detected, significant differences are marked by letters.

The reaction of S_I plants was similar, this stress impact also ended on the 48th d.a.v. but plants already reached pod development stage (BBCH 70).

Compensation of early stress was detected directly after stress impact (33rd d.a.v.) and reached a 3fold level as opposed to control on the 38th d.a.v.. At this point plants were budding (BBCH 52).

Both invertases showed compensation along a time scheme: the potential of compensation could be observed between 31st d.a.v. and 45th d.a.v, and after the 45th d.a.v..

Stress imposed at inflorescence emergence (S_I; start at 33rd d.a.v.) led to a direct stress response: activity did not decline for the following seven days as in the control.

The same reaction could be observed in the activity of the extracellular invertase.

A comparable compensation reaction of all three inverstases was observed in the the early stress variant even though stress impact already ended on 33rd d.a.v..

In the prolonged stress variant, activity of cytosolic and vacuolic invertase tended to follow the activity level in the control until 64th d.a.v.. The activity of the extracellular invertase was lower in comparison with the other two invertases up to 45th d.a.v., where activity increased rapidly, matching the level of control plants at 49th d.a.v.. At this point the control was in the stage of fruit development while the prolonged stress variant was still in inflorescence emergence.

Stress impact and compensation was observed by potential photosynthetic activity (Figure 5).

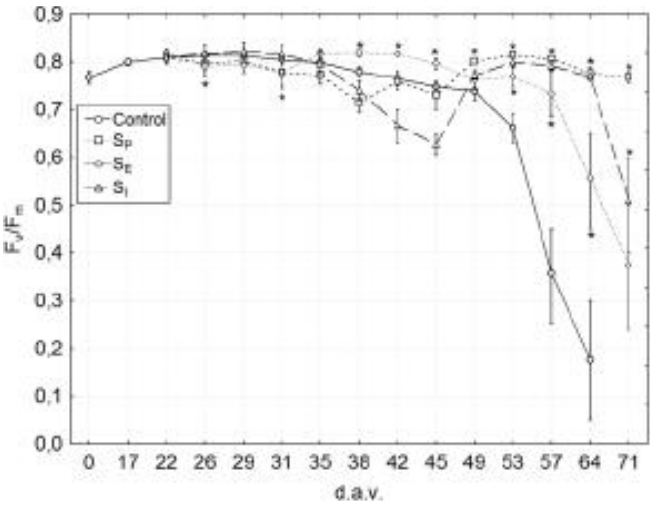

Figure 5 : Potential photosynthetic activity (maximum efficiency of photosystem II) influenced by drought stress (S_E = early stress, S_P = prolonged stress, S_I = inflorescence stress), significant differences to control are marked by asterisk.

Significant depression of activity during dry period was followed by a significant activity increase 5 days after the stress impact ended. Although stress impact endured till the 48th d.a.v. in the prolonged stress variant, potential photosynthetic activity increased to control level between 42th and 45th d.a.v.. Decreased activity levels at 71th d.a.v. corresponded to the delay of

ontogenetic development: control had the lowest activity (BBCH 76), followed by S_E and S_I (BBCH 72) and S_P (BBCH 61). Leaf senescence in control plants initiated on the 53th d.a.v., whereas the stress variants showed specific delayed leaf senescence, dependent on timing and duration of stress impact.

In all plants, corrected osmolality was similar between the 17th and the 45th d.a.v., and, peaked on the 29thd.a.v.. Activities of cytosolic, vacuolic and extracellular invertases were similar from the 17th to 31st d.a.v. with peaks on the 29th and 45th d.a.v. with exception of the extracellular invertase activity which did not have the second peak on the 45th d.a.v. but on the 49th d.a.v..

Net photosynthesis and transpiration reflected the present stress status by decreasing under stress impact within two days and increasing after stress within three days (data not shown).

Biomass

The dynamics of plant development can be reflected by biomass analysis. Here it could be observed that biomass in stress variants differed from control. In particular, S_P and S_I stress variants showed a depression in biomass (Figure 6). In early stressed plants a fast increase of biomass was detected in the recovery phase, resulting in biomass similar to control. Pod mass of control plants was 14 g (dry weight) per plant at harvest time (BBCH 89-90). In S_P-plants, however, pod biomass was decreased compared to control plants. Inflorescence stress had the highest impact on shoot biomass. However, pod biomass was yet similar to control. In this context a change in architecture of inflorescence of the S_P-variant could be observed: Development of main inflorescence was inhibited by drought stress; moreover, compensation during recovery was related to an intensified development of lateral inflorescence.

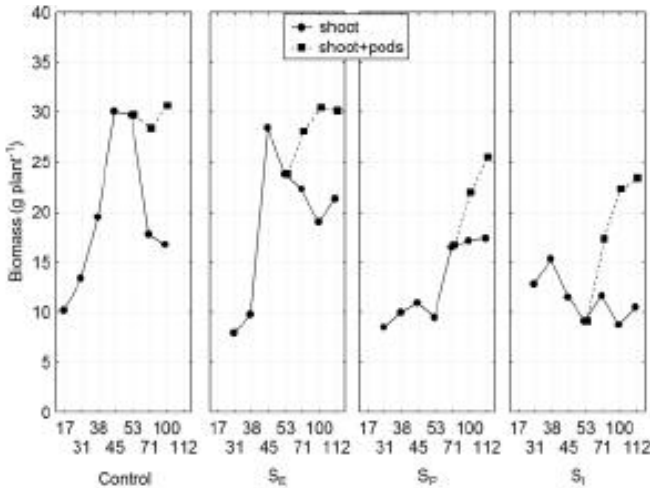

Figure 6 : Development of biomass influenced by dry periods (S_E = early stress, S_P = prolonged stress, S_I = inflorescence stress).

Mean pod dry mass of the four variants was highly correlated with the mean activity of extracellular invertase (R=0.99), net photosynthese (R=0.93) and moderately correlated with vacuolic invertase activity (R=0.80). Transpiration (R=0.87), RWC (R=0.87), and osmolality (R=-0.97) were correlated to shoot dry mass.

DISCUSSION

Impact of drought on invertase activities, induced osmolytes and seed biomass development under drought stress and its recovery in various ontogenetic stages was analyzed in oilseed rape.

Net photosynthesis, potential photosynthetic activity and transpiration showed a more or less immediate reaction to applied drought stress followed by a compensation reaction shortly after the end of stress impact. Corrected osmolality and the activities of extracellular, vacuolic and cytosolic invertases were found to be stress-independent at certain fixed time points.

Chlorophyll fluorescence and net photosynthesis reach the highest degree of depression during inflorescence stress. The more sensitive reaction of net photosynthesis seems to be based on a fast regulation of stomata at early stress or recovery phases. Light-saturated stomatal conductance generalizes the responses of many photosynthetic parameters to drought[17]. Bota et al.[18] found decreased Rubisco activity and a substantial decline of RuBP availability correlating with a complete dropdown of photosynthesis only during severe drought. Photosynthesis dropdown was also observed in all three stress variants in the present study. The strength of stress did not induce a water deficit compensatory production of osmolytic substances as one could expect. The increase of osmotic pressure through stress was an effect of water loss only; the corrected osmolality was similar to the control. Application of a prolonged pe-

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riod of stress reduces osmolytic substances, seen as a reduction of corrected osmolality. In this stress variant, only after being exposed to a further 6 day stress elongation, we found a production of osmolytes up to twice the level found in control plants (450 mmol kg^{-1}, data not shown). This active mechanism occurred between the 45th and 53rd d.a.v., shortly before plants died on the 54th d.a.v.. This is in accordance to simulated effects of prolonged drought on photosynthesis, non-structural carbon compounds and respiration[19]. In McDowell's simulation the pool of non-structural carbohydrates is constant for the first 20 days of drought impact. Therefore, assuming that soluble carbohydrates mainly function as osmolytes, this finding is in concordance with our results, where the corrected osmolality equals the control. The simulation cited above shows an increase in soluble carbohydrates for the following 10 days, which can also be seen by means of corrected osmolality in our study. This rapid production of osmolytes however does not prevent the plant from reaching a critical tipping point in mortality. This can be explained by a strong effect on carbohydrate transport, utilization and mobilization[19]. Therefore, plants starve to death rather than dying of water deficit. Analysis of further parameters revealed an additional time effect, which was found to be independent from ontogenetic stage, stress impact or recovery. These points in time, 29th, 31st, 42th, 45th and 53th d.a.v., were found through the parameters corrected osmolality, and invertase activities. At these points in time, the control plants reached the following ontogenetic stages: on 29th, 31th d.a.v. inflorescence emergence (BBCH 50-51), on 42nd/45th d.a.v. flowering declining (BBCH 67), on 49th d.a.v. development of fruit (BBCH 70) and on 53th d.a.v. 40% of pods have reached final size (BBCH 74). Therefore, these points in time all mark transitions between two main stages of plant growth with exception of the 53rd d.a.v., which marks the end of the first half in fruit development. Genetic analysis shows a correlation of distinct quantitative trait loci (QTL) to bolting (BBCH3), budding (BBCH 5) und flowering time (BBCH 6) in Brassica[20-22]. We assume that it is these genetic regulations of budding and flowering, which are independent from delayed plant development caused by the impact of drying stress, that are detected by above parameters. No hint was found for bolting, which could be a fast process not detectable in our 2-3 measurement day interval. Bolting could also be linked to flowering insofar as flowering can only occur if bolting precedes[21].

Dodd et al.[23] and Robertson et al.[24] demonstrated that a large proportion of transcripts involved in hormonal metabolism, catabolism, and signaling are regulated by a circadian clock. Such a clock seems to exist also for ontogenetic development. Because of a strong vernalisation all control plants flowered nearly simultaneously in our study. Therefore the ontogenetic clocking of individual plants was synchronized at certain days after vernalisation, even if delayed ontogenesis took place in the stress variants. These ontogenetic time points are characterized by defined values of corrected osmolality and invertase activities. On the level of the circadian clock, interactions between circadian clock, metabolisms and stress reactions, e.g. the pronounced formation of axialliary buds, have been multiply described[25-27]. However, we found a pronounced formation of axillary flower buds only under stress condition of dry period started in the inflorescence stage. As reviewed by Imaizumi[28] in arabidopsis, mobile signals and flowering signals are transported from leaves to stem[29, 30]. Dry periods can inhibit such transport, and can change the distribution of mobile signals within shoot meristems[29].

Inflorescence stress seems to be overcompensated. Reduction of shoot biomass by 25% in conjunction with a sustained pod mass illustrates the compensating power of upregulated invertases, i.e. the up regulation of extracellular invertase during and after stress to about twice the amount as in control in the time frame between the fixed time points 31st and 49th d.a.v.. However the compensatory invertase activity does not exceed the maximum level of regular invertase activity measured in the control at 29th d.a.v.. Thus, compensation seems to be permuted through prolonged high activitiy. Cumulative or averaged extracellular invertase activity is highly correlated to pod dry mass in all variants, as found also in the variety "Baldur"[14]. Activities from single points in time do not correlate to biomass parameters, as also described by Sulpice et al.[31].

Early stress impact on plants of the variety "Titan"

is compensated in regard of shoot biomass by flowering 7 days later and a prolonged fruit development by 7 days, yet stress is not sufficiently compensated regarding pod biomass loss (-30%). As we mentioned in an earlier study using the oilseed rape variety "Baldur" under early stress[14], a more flexible date of mean flowering, like in the "Titan" cultivar, would compensate stress impact on dry weight of shoots (-20%) also in the "Baldur" variety. The more relevant compensation of pod biomass loss, which was 30% dry weight of pods in both varieties, seems to be related to the activity of extracellular invertase, which is downregulated after the 45th d.a.v., even though a compensation by up regulation of all three invertase activities before this point in time occurs, albeit the average activity of the extracellular invertase over the course of ontogeny is lower than in control. Under prolonged stress, the absence of a compensation reaction and lower activity than in control led to a biomass loss in pods of 50% dry matter even though dynamics in activity were similar to control, yet on a lower level.

The present study demonstrates that an ontogenetic time table induces reactions of corrected osmolality and invertase activities. This limits compensation of delayed development to ontogenetic fixed time windows, despite existing regulatory mechanisms which could in theory be used by plants to fully compensate the stress impact. Further research may bring forth more detailed knowledge on the importance of ontogenetic timing in terms of reproduction and yield.

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