



Properties of root water transport in canola (*Brassica napus*) subjected to waterlogging at the seedling, flowering and podding growth stages

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Abstract

Aims The objective was to clarify the role of root water transport in waterlogged canola plants. We examined the hypothesis that waterlogging triggers root suberization and lignification, which reduces the effectiveness of apoplastic bypass and increases the dependence of roots on the aquaporin-mediated water transport, and that this effect varies depending on the growth stage.

Methods *Brassica napus* plants at the seedling, flowering and podding growth stages were waterlogged for up to 8 days. Growth, gas exchange, leaf water potentials, and root hydraulic conductance were measured in addition to relative contributions of the aquaporin-mediated and apoplastic root water transport, gene expression levels of plasma membrane intrinsic proteins (*BnPIPs*), and lignin and suberin deposition in roots.

Results Waterlogging decreased dry weights, gas exchange, leaf water potentials and root hydraulic conductivity more in plants at the seedling stage than other growth stages. It also accelerated root suberization and lignification resulting in an increase of a relative

contribution of aquaporin-mediated water transport. The transcript levels of *BnPIPs* increased with an increasing duration of waterlogging.

Conclusions Effects on root water transport played an important role in the sensitivity of canola seedlings to waterlogging. Increased relative contribution of aquaporin-mediated water transport in waterlogged plants was accompanied by enhanced root suberization and lignification. The evidence points to the importance of maintaining functional aquaporins in plant survival of waterlogging.

Keywords Apoplastic pathway · Aquaporins · Gas exchange · Root hydraulic conductance · Water relations

Introduction

Waterlogging is a major constraint to plant growth and survival across the globe, especially in the areas with high precipitation and poor soil drainage. It is also a common factor limiting the productivity of many agricultural crops including canola (*Brassica napus*) (Perboni et al. 2012). Canola is one of the most important oilseed crops in the world including Canada. The crop is seeded shortly after the snowmelt in Canada and as a rice rotation crop in paddy fields in China (Xu et al. 2015b), which leads to high risks of waterlogging. Distinct yield reductions in canola may occur after 3 to 30 days of flooding, depending on other environmental stress factors and the developmental stage of plants (Habibzadeh et al. 2012).

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Waterlogging reduces oxygen availability to plants. Oxygen diffusivity in water is approximately 10,000 times lower than it is in air, and the flux of O₂ in soil is approximately 320,000 times lower when water fills the soil pores (Armstrong and Drew 2002). This leads to root hypoxia, which triggers declines in metabolic processes and induces rapid functional and structural alterations in waterlogged plants (Mielke and Schaffer 2010; Tan and Zwiazek 2019). Upset plant water balance has been often reported in waterlogged plants and explained as effects of hypoxia on root structure and on aquaporin-mediated root water transport (Jitsuyama 2017; Kreszies et al. 2018). Roots are the sites of greatest resistance to water flow in most plants (Steudle and Peterson 1998) and, in addition to waterlogging, root hydraulic conductivity is also commonly altered by other environmental stresses including salinity, drought, and low soil temperature (Lee et al. 2012; Grondin et al. 2016; Meng and Fricke 2017; Vaziriyeganeh et al. 2018).

Radial water flow in roots follows the apoplastic and cell-to-cell (across cell membranes and through plasmodesmata) pathways (Knipfer and Fricke 2010). The apoplastic pathway predominates when hydrostatic gradients are created by transpiration while cell-to-cell pathway, which is largely mediated by aquaporins, plays a critical role under stress (Steudle and Peterson 1998; Maurel et al. 2008). However, the dynamic changes of these pathways have not been thoroughly investigated in waterlogged plants. Relative contributions of apoplastic and cell-to-cell pathways may substantially change depending on the type and duration of environmental stress in different plants (Steudle and Peterson 1998; Martínez-Ballesta et al. 2003; Henzler et al. 2004). Roots can adjust their transport properties in response to stress through structural modifications altering the apoplastic pathway (Bárzana et al. 2012) or through changes in the properties of aquaporin-mediated cell-to-cell water transport (Barberon et al. 2016). Since waterlogging induces an accumulation of lignin and suberin (Abiko et al. 2012), their deposition in roots could potentially decrease root hydraulic conductivity. Cell-to-cell pathway is regulated mainly by the abundance and activity of aquaporins, therefore, it may also be affected by the acidification of cytoplasm as well as by impeded membrane trafficking and phosphorylation processes that are likely to occur as a result of waterlogging (Kamaluddin and Zwiazek 2002; Maurel et al. 2015). It is also plausible that waterlogging could affect the aquaporin-mediated transport of other

molecules than water including O₂, CO₂, NO, H₂O₂ and lactic acid (Uehlein et al. 2012; Tian et al. 2016; Zwiazek et al. 2017; Tan et al. 2018) and contribute to stress tolerance.

Plants vary in their sensitivity and may use different strategies to tolerate waterlogging during different stages of growth (Ren et al. 2017; Romina et al. 2014). Therefore, in the present controlled-environment study, we examined the effects of waterlogging on root water flow properties in relatively hypoxia-sensitive canola (*Brassica napus*) plants during the seedling, flowering, and podding stages of growth. We used the tracer dye, light green SF yellowish and silver nitrate to determine relative contributions of apoplastic and cell-to-cell pathways in aerated and waterlogged plants.

The objectives of the present study were to examine the effects of waterlogging on root water transport pathways at different growth stages in canola to clarify their role in plant responses to root hypoxia. We examined the hypothesis that waterlogging triggers root suberization and lignification, which reduces the effectiveness of apoplastic bypass and increases the dependence of roots on the aquaporin-mediated water transport. Therefore, maintaining the functionality of aquaporins is an important element for plant tolerance to waterlogging.

Materials and methods

Plant material and treatments

Canola (*Brassica napus* L. cv Westar) seeds were surface-sterilized with 70% ethanol for 2 min followed by 20% (v/v) commercial bleach (1% [v/v] sodium hypochlorite) for 30 min. The seeds were germinated in half-strength Murashige and Skoog (MS) medium at 20 °C. Shortly after germination, the seedlings were moved into autoclaved peat moss/vermiculite (2:1) in sterilized 1 L (10 × 10 × 10 cm) square plastic pots. The seedlings were grown in a controlled environment growth room with 16 h photoperiod, 22/18 °C (day/night) temperature, 400 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD), and 50–60% relative humidity. The seedlings were watered every other day and fertilized weekly with 50% modified Hoagland's solution (Epstein 1972). When 4-week (seedling stage), 8-week (flowering stage) and 11-week (podding stage) old, 16 plants from each stage were randomly divided into the control and waterlogging treatment groups. For

the waterlogging treatment, for each growth stage, six pots with plants (one plant per pot) were placed in each of the five replicated 40 L plastic tubs (60 × 40 × 20 cm) filled with water up to 2 cm above the top of the pot. The control (also referred to as aerated) plants were grown in well-drained soil throughout the experiment (Fig. S1).

Measurements of gas exchange, leaf water potentials and dry weights

After 2, 5, and 8 days of waterlogging, net photosynthesis rate (P_n) and transpiration rate (E) were measured approximately from 9:00 to 12:00 h using a Licor-6400 portable photosynthesis system with a 2 × 3 cm² leaf chamber (LI-COR, Lincoln, NB, USA). The reference CO₂ concentration was set to 400 μmol mol⁻¹; the flow rate was 200 μmol s⁻¹. The leaf chamber temperature was maintained at 20 °C, and PPFD was 400 μmol m⁻² s⁻¹ of the red-blue light spectrum. Six plants from the control group and six plants from the waterlogged group were randomly picked and three fully expanded uppermost leaves from each plant were measured. The averages of the three leaves from each plant was taken to calculate the mean gas exchange values for six plants from the each control and waterlogged groups ($n = 6$). After the measurements, the leaves were excised and immediately placed in a Scholander pressure chamber (PMS instruments; Corvallis, OR, USA) for the measurements of leaf water potential (Ψ_{leaf}) (Scholander et al. 1965; Wan and Zwiazek 1999) ($n = 6$). Dry weights of total plants were determined after oven drying at 80 °C for 48 h ($n = 6$). Leaf areas were calculated using Sigmascan Pro 5.0 software (Systat Software, San Jose, CA, USA).

Measurements of root hydraulic conductance (K_r) and apoplastic and cell-to-cell water transport pathways

After 2, 5, and 8 days of waterlogging, root hydraulic conductance (K_r) was measured with a hydrostatic pressure method (Wan and Zwiazek 1999; Wan et al. 2001). A 0.25 L glass container containing half-strength Hoagland's solution was placed in a pressure chamber (PMS Instruments, Corvallis, OR, USA). Roots were excised above the root collar, immersed in Hoagland's solution in the container and sealed in the pressure chamber with the short part of the stem protruding above the pressure chamber lid. The measurements of root water flow rates were carried out by determining the

volume of excluded sap after pressurizing the chamber to 0.3, 0.4, 0.5 MPa for 2 min (Wan and Zwiazek 1999). Linear regression of the applied pressure and flow rate was used to obtain a slope of the relationship which represented K_r (Fig. S2).

The light green SF yellowish dye (light green SF yellowish; Sigma-Aldrich Chemical) was used to determine the relative contributions of apoplastic and cell-to-cell pathways. (López-Pérez et al. 2007). Light green SF yellowish moves apoplastically and is not transported across the plasma membrane (Martínez-Ballesta et al. 2003). Detopped root systems were immersed in 250 μmol L⁻¹ light green SF yellowish solution inside a Scholander pressure chamber for 5 min before raising pressure to 0.4 MPa in the chamber. The xylem exudate was collected for two minutes and the dye concentration were immediately measured with a spectrophotometer (Genesys 10 S-UV-VIS, Thomas Scientific, NJ, USA) at 630 nm (Bárzana et al. 2012). The percentage of apoplastic pathway contribution was calculated from the ratio between dye concentrations of the collected xylem sap and the applied solution.

We also used an aquaporin water transport inhibitor, silver nitrate (Tharanya et al. 2018), to determine changes in the aquaporin-mediated water transport of waterlogged plants compared with well-aerated control. Following K_r measurements, the roots were immersed in 400 mM AgNO₃ for 3 h (Tharanya et al. 2018), and K_r was determined again as described above. The contribution of aquaporin-mediated water transport to hydraulic conductance was calculated from the difference in K_r before and after the addition of AgNO₃.

RNA transcription profiling

The relative transcript abundance was analyzed for six *PIP* genes cloned from *B. napus* (Ge et al. 2014): *BnPIP1;1* (NCBI accession number NC_027760), *BnPIP1;2* (NC_027761), *BnPIP1;3* (NW_019168728), *BnPIP2;2* (NC_027769), *BnPIP2;5* (NC_027765), *BnPIP2;7* (NC_027757) (Primer sequences are shown in Table S1). After 2, 5, and 8 days of treatment, 3–5-cm-long distal root segments from 4 plants in each treatment group were excised and quickly frozen in liquid nitrogen before being transferred to the -80 °C freezer. The samples were ground with a mortar and pestle in liquid nitrogen and total RNA extracted with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA USA). First-strand complementary DNA (cDNA) synthesis and the real-time quantitative PCR (qRT-PCR)

were conducted as earlier described (Xu et al. 2015a). For qRT-PCR, three technical replicates of each cDNA sample were analyzed for each of the four biological replicates ($n = 4$). Relative transcript abundance was calculated using the $2^{-\Delta\Delta C_t}$ method. The relative transcript fold change of *PIPs* was normalized against geometric mean of the C_T value of two reference genes *BnACTIN7* (AF_111812) and *BnPPR* (XM_013831783).

Root anatomy

Distal root segments ($n = 5$) from each treatment were prepared for microscopy according to Roschztardtz et al. (2009). Root segments around 3–5 cm from the end were fixed in formalin-acetic acid-alcohol (FAA) solution. After fixation, the root segments were dehydrated in an ethanol series, placed in toluene. Fixed root segments were embedded in paraffin and sectioned with a microtome (model RM2125 RTS, Leica; Solms, Germany). Lignin autofluorescence was visualized following UV excitation at 330 nm to 380 nm with a fluorescent microscope (Carl Zeiss; Jena, Germany) (Donaldson and Radotic 2013; Jia et al. 2015). The light intensity of lignification was determined with Image J software (<https://imagej.nih.gov/ij/>) ($n = 5$ images per group) and quantified as previously described (Yamaguchi et al. 2010). For suberin visualization, the protocol developed by Lux et al. (2005) was adopted. Briefly, thin paraffin sections were incubated in a freshly prepared solution of Sudan 7B (0.01% w/v, in lactic acid) at room temperature for 30 min and then rinsed in 90% ethanol (two baths of 1 min each). Samples were then mounted on slides in 50% glycerol and observed under ZEISS AXIO compound light microscope (Carl Zeiss; Jena, Germany). Suberin was quantified in microscopic sections as described by Kreszies et al. 2019.

Statistical analysis

All data were analysed using the SPSS 18.0 statistical package (SPSS Inc., Chicago, IL, USA). Statistically significant differences between well aerated control and waterlogged plants for each growth stage were determined using the one-way ANOVA followed by Tukey's test ($P \leq 0.05$).

Results

Growth parameters, gas exchange and leaf water potentials

Total plant dry weights were significantly reduced compared with control plants after 8 days of waterlogging at the seedling and flowering stages (Fig. 1a, b). Compared with aerated controls, leaf areas were smaller in waterlogged plants at the seedling and flowering stages after 8 days of waterlogging and after 2 days of waterlogging at the podding stage (Table 1). Defoliation was observed after 8 days of waterlogging at both seedling and flowering stages (Table 1). Plant heights were affected after 8 days of waterlogging at the seedling stage, whereas root lengths were reduced when measured after 8 days of waterlogging at the seedling stage compared with aerated control plants (Table 1).

Net photosynthesis (P_n) and transpiration (E) rates had the highest values at the seedling stage, following by flowering and podding stages (Fig. 2). Compared with control plants, P_n was significantly reduced after 5 days waterlogging at the seedling and flowering stages and 8 days of waterlogging at all growth stages (Fig. 2a–c). Waterlogging also significantly decreased E after 5 and 8 days at the seedling and flowering stages (Fig. 2d, e). However, no marked reductions in E were observed at the podding stage (Fig. 2f).

Compared with well-aerated control, leaf water potentials (Ψ_{leaf}) were sharply reduced at the seedling stage after 2 and 8 days of waterlogging (Fig. 3a). Small reductions in Ψ_{leaf} were also measured after 2 and 8 days of waterlogging at the flowering stage and after 2 days of waterlogging at the podding stage (Fig. 3b, c).

Root water transport

Waterlogging reduced root hydraulic conductance (K_r) compared with control plants at durations at seedling stage and 5, 8 days at flowering stage. Mild reductions were observed in the podding stage (Fig. 3d–f). The largest, several-fold decreases in K_r were measured at the seedling stage when waterlogged for 2 days (Fig. 3d).

The light green SF yellowish dye concentration in the root xylem sap was lower in waterlogged compared with control plants at all growth stages, indicative of a greater contribution of transmembrane, relative to apoplastic, water transport pathway during

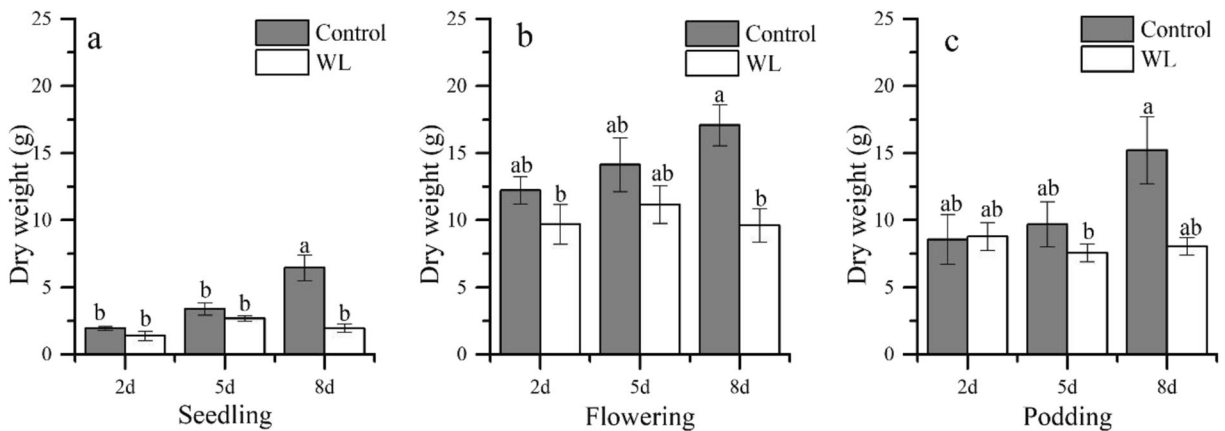


Fig. 1 Plant dry weights of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage (a). F2, F5, F8 are 2, 5, and 8 days of waterlogging at the

flowering stage (b). P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage (c). Means \pm SE are shown ($n = 6$). Different letters above the bars indicate a difference determined by one-way ANOVA ($P \leq 0.05$; Tukey's test)

waterlogging. The dye concentration declined in plants with waterlogging over time starting at day 2 at the seedling and podding stages and day 8 at the flowering stage (Fig. 4a–c).

K_r was reduced in both aerated and waterlogged roots treated with AgNO_3 , and the decreases varied between the growth stages and waterlogging treatment. In aerated control plants, the reductions were relatively higher at the seedling and flowering stages compared with the podding stage (Fig. 4d–f). The inhibition was greater in waterlogged compared with aerated control plants, especially at the seedling and flowering stages (Fig. 4d, e). The K_r reductions in AgNO_3 treated plants deepened with the waterlogging duration at all stages (Fig. 4d–f).

Relative transcript abundance of *PIP* aquaporins

The relative transcript abundance, which indicates the gene transcriptional levels relative reference genes, of 6 *BnPIP*s aquaporins was quantified using qRT-PCR in the distal root segments of waterlogged and control plants. After waterlogging for 2 days, plants at the seedling stage showed sharp increases in *BnPIP2;7*, while the expression level of *BnPIP2;2* was upregulated after 5 days of waterlogging and *BnPIP1;3* expression level was upregulated after 8 days of waterlogging (Fig. 5). However, *BnPIP1;1* was downregulated after 2 days of waterlogging at the seedling stage (Fig. 5). At the flowering stage, most of the *BnPIP*s expression levels were downregulated in waterlogged plants, including *BnPIP1;1*, *BnPIP1;3*, and *BnPIP2;5* (Fig. 5).

However, *BnPIP1;2* was upregulated over time and peaked after 8 days of waterlogging (~102-fold). *BnPIP2;7* expression level increased with the duration of waterlogging at the flowering stage (Fig. 5). At the podding stage, *BnPIP1;2*, *BnPIP1;3* and *BnPIP2;5* were upregulated after 8 days of waterlogging (Fig. 5).

Effect of waterlogging on suberization and lignification in roots

Suberization of endodermis increased with increasing plant age starting at the inner side of the endodermis while lignification was not evident (Fig. 6a, b). Suberization also increased in the waterlogged plants. The suberization induced by waterlogging was mostly observed at the seedling stage starting at 2 days following waterlogging and after 8 days of waterlogging at the flowering and podding stages (Fig. 6a). To quantify the suberization level, the numbers of suberized cells in the root endodermis were calculated. The number of suberized cells in waterlogged roots was about twice as high as compared with the aerated control plants after 8 days of waterlogging at the seedling stage (Fig. 7a). When waterlogged for 8 days of flowering and podding stage, waterlogged plants had significantly more suberized cells than aerated plants (Fig. 7b, c).

The greatest lignin deposition was observed after 5 and 8 days of waterlogging at the seedling stage and 5 days of waterlogging at the flowering stage (Fig. 7d–f). Plant stage of growth did not influence the degree of lignification (Fig. 6b). The lignin autofluorescence

Table 1 Plant heights, numbers of leaves, root lengths and leaf areas in aerated control (C) and waterlogged (WL) canola and in plants waterlogged for 2, 5, and 8 days at the three growth stages. Means \pm SE are shown (n = 6). Letters indicate a difference determined by one-way ANOVA ($P \leq 0.05$; Tukey's test)

Stage	Height (cm)	Leaf number		Root length (cm)		Average leaf area (cm ²)			
		C	WL	C	WL	C	WL		
Seedling	2d	17.98 \pm 1.02bc	16.46 \pm 0.81c	6.81 \pm 0.21ab	5.84 \pm 0.24b	22.00 \pm 1.89ab	26.34 \pm 2.10a	115.84 \pm 15.49bc	77.16 \pm 12.32c
	5d	19.00 \pm 1.11b	18.46 \pm 1.38ab	7.25 \pm 0.35ab	7.25 \pm 0.25ab	27.07 \pm 0.86a	27.61 \pm 2.89a	148.05 \pm 9.59cb	99.78 \pm 9.35bc
	8d	22.67 \pm 0.84a	16.42 \pm 1.19bc	8.50 \pm 0.26a	6.75 \pm 0.37b	27.98 \pm 1.02a	15.96 \pm 3.62b	176.92 \pm 11.71a	82.47 \pm 9.75c
Flowering	2d	91.83 \pm 6.64ab	71.60 \pm 5.92b	12.58 \pm 0.70a	12.00 \pm 0.73ab	26.60 \pm 1.32a	30.85 \pm 2.28a	121.27 \pm 13.13a	89.64 \pm 8.90ab
	5d	89.33 \pm 7.36ab	72.25 \pm 3.42b	10.08 \pm 0.40b	10.25 \pm 0.43b	31.10 \pm 1.31a	31.13 \pm 6.63a	93.13 \pm 6.39ab	65.941 \pm 7.13bc
	8d	97.89 \pm 6.08a	85.25 \pm 5.45ab	11.00 \pm 0.50ab	6.75 \pm 0.25c	26.68 \pm 1.39a	28.45 \pm 2.61a	101.65 \pm 9.02a	42.99 \pm 4.46c
Podding	2d	115.70 \pm 11.75a	108.6 \pm 5.05a	7.67 \pm 0.84a	6.09 \pm 0.37ab	28.88 \pm 1.77a	26.05 \pm 1.26a	60.70 \pm 6.55a	32.00 \pm 3.76b
	5d	120.44 \pm 10.27a	116.78 \pm 8.60a	4.86 \pm 0.91abc	3.33 \pm 0.78bc	29.38 \pm 1.65a	27.63 \pm 1.15a	46.81 \pm 7.10ab	28.45 \pm 4.75b
	8d	115.81 \pm 9.55a	110.20 \pm 8.31a	3.38 \pm 0.69bc	2.13 \pm 0.55c	26.00 \pm 1.61a	25.25 \pm 1.94a	44.57 \pm 11.41ab	29.52 \pm 6.23b

intensity at the seedling and flowering stage was slightly higher in waterlogged plants, compared with control plants after 2 days of waterlogging but not significant (Fig. 7d, e). The light intensity difference between waterlogged and control plants were not significant during the podding stage (Fig. 7f).

Discussion

Our study demonstrated that waterlogging differently affected root water transport properties as well as growth, gas exchange and leaf water potentials depending on the growth stage of plants. Since efficient delivery of water to shoots is essential for maintaining gas exchange and growth processes, overcoming the challenges of waterlogging to root water transport may be key to understanding plant tolerance of this challenging condition.

There is abundant literature demonstrating the importance of root water transport in plant tolerance of a variety of abiotic stresses and the role of aquaporins in these responses (Chaumont and Tyerman 2014; Kapilan et al. 2018). Radial water transport in roots follows the route of least resistance, which is a combination of apoplastic and aquaporin-mediated cell-to-cell pathways (Steudle and Peterson 1998; Steudle 2001). It has been previously demonstrated that root hypoxia induced by waterlogging inhibits the aquaporin-mediated root water transport due to the cellular acidosis and depletion of ATP that is required for protein phosphorylation (Kamaluddin and Zwiazek 2001; Kamaluddin and Zwiazek 2002; Tournaire-Roux et al. 2003; Bramley et al. 2007).. Waterlogging can also alter root structure with the consequences to apoplastic pathway. Increased suberization (Tylová et al. 2017) and lignification (Nguyen et al. 2016) have been reported in the roots of some plants in response to waterlogging, could have major consequences to water flow resistance in the roots. It is also well established that waterlogging tolerance varies at a different plant age and stage of development (Xu et al. 2015b). Our study sheds more light on the role of root water transport in this process. Longer waterlogging durations altered the contributions of apoplastic and aquaporin-mediated water transport at each growth stage. Notably, plants at the seedling stage relied more on the aquaporin-mediated water transport for their extensive water requirement and rapid vegetative growth comparing with other growth stages. In

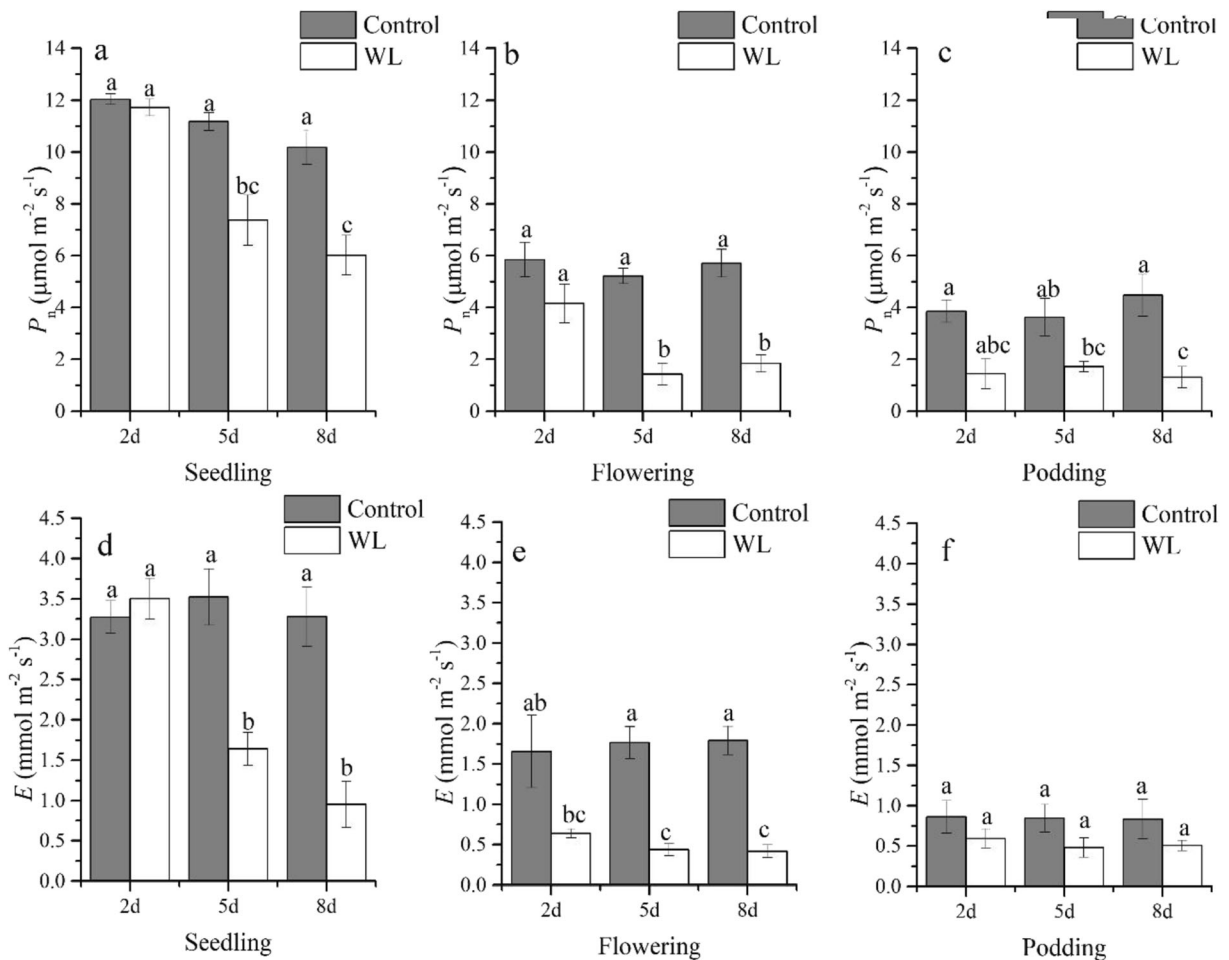


Fig. 2 Plant net photosynthesis (P_n) and transpiration rate (E) of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage (a, d). F2, F5, F8 are

2, 5, and 8 days of waterlogging at the flowering stage (b, e). P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage (c, f). Means \pm SE are shown ($n = 6$). Different letters above the bars determined by one-way ANOVA ($P \leq 0.05$; Tukey's test)

addition, their roots contained relatively little suberin and lignin which would otherwise restrict apoplastic water transport. Furthermore, we found that canola plants at the seedling stage were less flooding tolerant compared with the flowering and podding stages.

Waterlogged plants at the seedling stage exhibited the strongest, while those at the podding stage displayed the weakest, declines in the total plant weights, P_n and K_r . This is consistent with the previous studies, which showed that changes of morphological traits under waterlogging at the seedling stage among 25 varieties of *B. napus* were greater compared with other growth stages (Xu et al. 2015b; Zou et al. 2014; Habibzadeh et al. 2013). It is noteworthy, that despite the larger root systems in aerated plants at the flowering and podding

stages, their K_r was lower compared with the plants at the seedling stage, likely due to a higher proportion of older roots. Waterlogging resistance at different developmental stages appears to be strongly linked to the water demand as root water transport capacity is sharply reduced by the hypoxic conditions. In addition to slower growth rates, partial defoliation that commenced at the flowering stage and resulted in a decrease in plant dry weights, also contributed to the lower water demand, especially for plants at the podding stage.

As expected, both Ψ_{leaf} and K_r support the notion of high water demand linked to high growth rates characteristic of young plants. This is likely one of the key factors that makes seedlings more sensitive to waterlogging. Additionally, mature plants are likely to

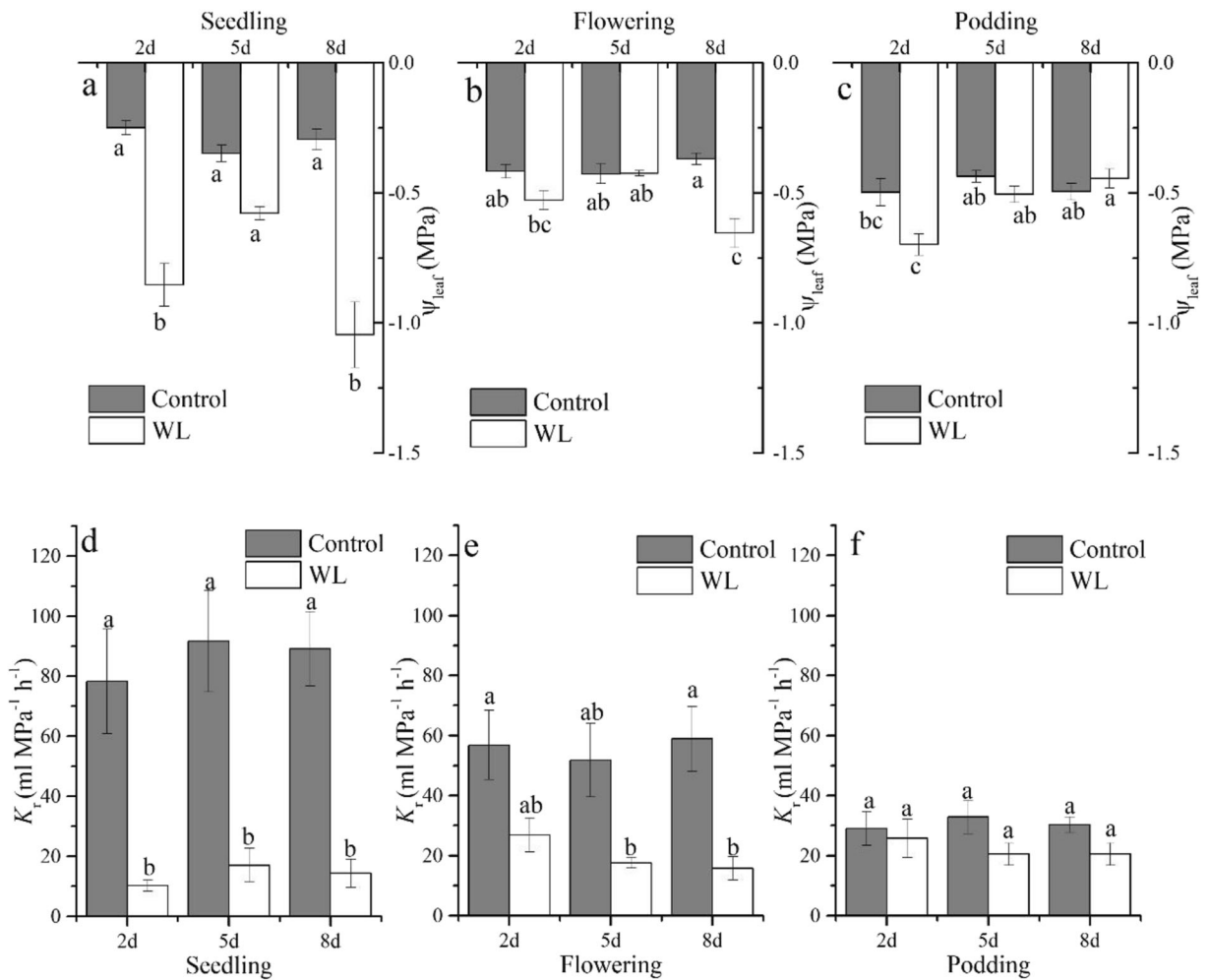


Fig. 3 Leaf water potential (Ψ_{leaf}) and root hydraulic conductivity (K_r) of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage (a, d). F2, F5, F8

are 2, 5, and 8 days of waterlogging at the flowering stage (b, e). P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage (c, f). Means \pm SE are shown ($n=6$). Different letters above the bars indicate a difference determined by one-way ANOVA ($P \leq 0.05$; Tukey's test)

have greater stored carbon resources (Groeneveld and Voeselek 2003) that can be exploited for fuelling fermentation and lowering osmotic potential (Huber et al. 2012). The Ψ_{leaf} declined in plants after 2 days of waterlogging and, similarly to other measured parameters, it was affected more at the seedling stage compared with the flowering and podding stages. Depending on the growth stage, a partial or full recovery of Ψ_{leaf} was observed after 5 days of waterlogging due to reductions in E . However, the E decreases were not sufficient to prevent subsequent declines of Ψ_{leaf} after 8 days of waterlogging in plants at the seedling and flowering stages. The decreases in K_r were profound and occurred

rapidly at the seedling stage and were relatively mild in plants at the flowering and podding stages.

The adjustment of K_r has been often reported for plants exposed to root hypoxia (Kamaluddin and Zwiazek 2002; Rasheed-Depardieu et al. 2015; Jitsuyama 2017) and attributed to the effect on aquaporin function and a switch between apoplastic and cell-to-cell pathways (Siemens and Zwiazek 2003). Symplastic and transmembrane pathways are together referred to as cell-to-cell pathway since they are experimentally difficult to separate (Kjellbom et al. 1999). Apoplastic water transport is commonly affected by alterations of root structure (Kreszies et al. 2019) while changes in cell-to-

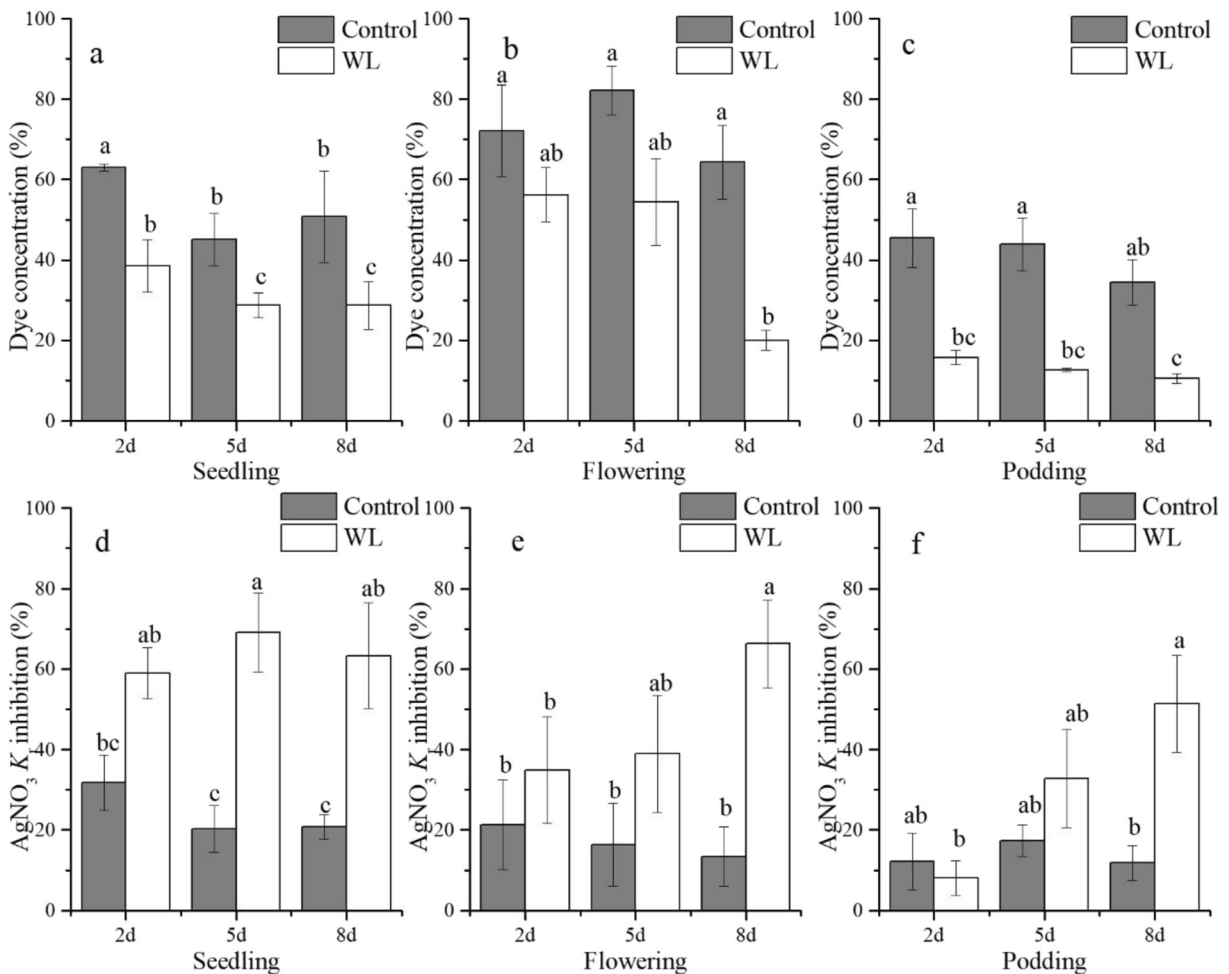


Fig. 4 Dye concentration (Light green SF yellowish) and AgNO_3 K_r inhibition of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage (**a, d**).

F2, F5, F8 are 2, 5, and 8 days of waterlogging at the flowering stage (**b, e**). P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage (**c, f**). Means \pm SE are shown ($n=6$). Different letters above the bars indicate a difference determined by one-way ANOVA ($P \leq 0.05$; Tukey's test)

cell pathway is mainly linked to rapid fluctuations of aquaporin gating and expression (Maurel et al. 2015). Our results of apoplastic dye light green SF yellowish and aquaporin water transport inhibitor AgNO_3 demonstrated that apoplastic pathway was dominant in aerated plants at the flowering stage and decreased after waterlogging for 5 and 8 days. The aquaporin mediated transport measured with aquaporin inhibitor AgNO_3 indicated that K_r reductions after AgNO_3 treated plants deepened with the waterlogging duration at all stages. This suggest that the ratio of apoplastic to cell-to-cell decreased in waterlogged plants following increased waterlogging duration at each stage. Short duration of waterlogging was mild stress to canola and longer

duration exerted severe stress. Longer waterlogging duration exerted a greater inhibition of apoplastic transport within each stage which is likely due to the gradual decreased E and cell wall modification (Ranathunge et al. 2016; Kreszies et al. 2019). If apoplastic pathway is blocked, water follows the less resistant cell-to-cell pathway regulated by aquaporins to reach the apoplast of the stele. The high expression of aquaporins with high water permeability can improve the radial hydraulic conductance and confer an efficient mechanism of regulating K_r (Bramley et al. 2007).

We determined the transcript abundance of *PIP1s* and *PIP2s* in distal root segments where aquaporins are the most abundant (Gambetta et al. 2013). Since

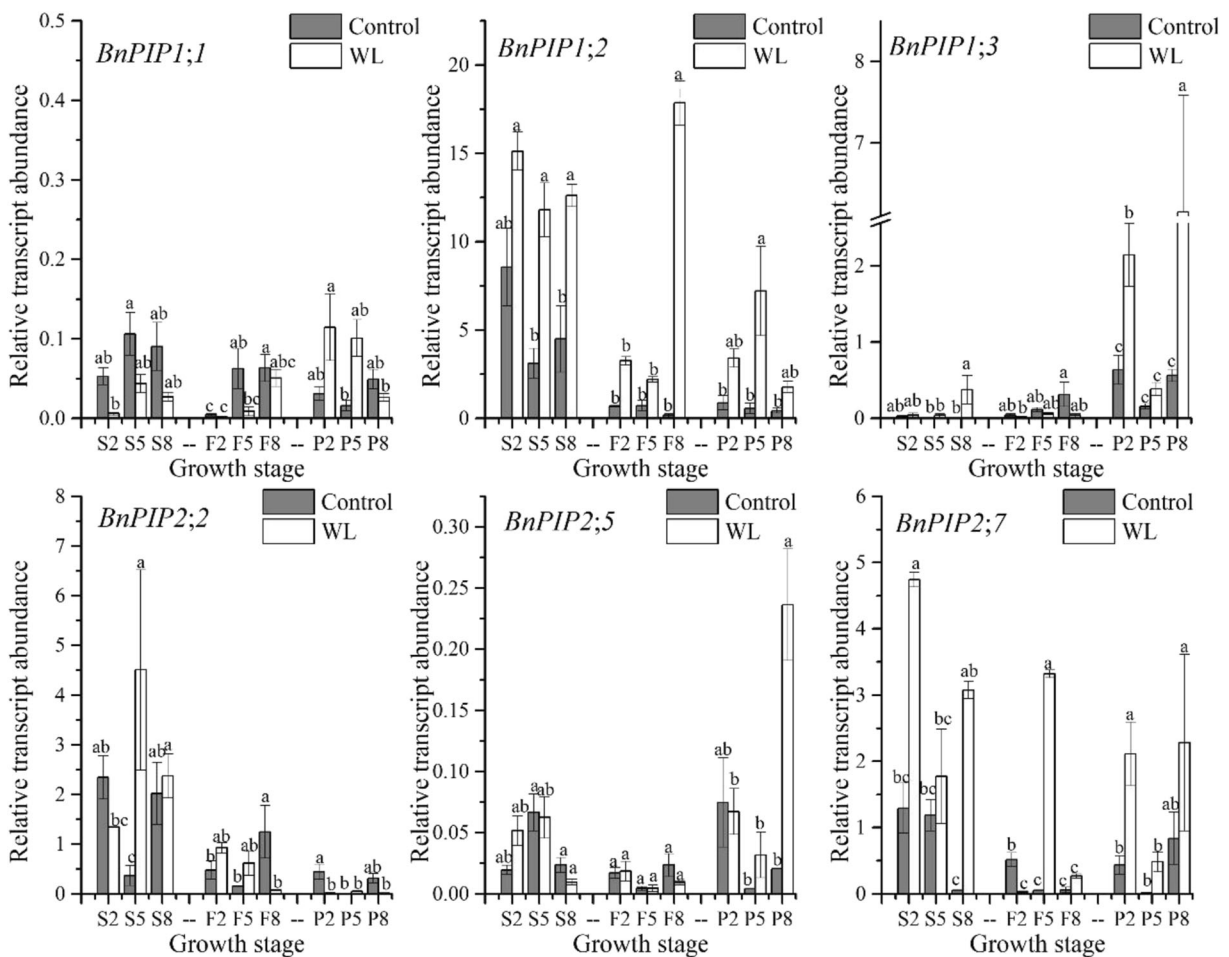


Fig. 5 Aquaporin (*BnPIPs*) relative expression level of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage. F2, F5, F8 are 2, 5,

and 8 days of waterlogging at the flowering stage. P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage. Means \pm SE are shown ($n = 4$). Different letters above the bars indicate a difference determined by one-way ANOVA ($P \leq 0.05$; Tukey's test)

protein abundance is primarily determined by the transcript levels (Pfannschmidt et al. 1999), *PIP* gene expression is an important mechanism that is used to control membrane water permeability (Maurel et al. 2008; Macho-Rivero et al. 2018). The results indicated that some of the *BnPIPs* were downregulated in response to 2 days of the waterlogging treatment which was consistent with previous studies (Rasheed-Depardieu et al. 2015). However, several *BnPIPs* showed a pronounced upregulation after two days of waterlogging. Rapid changes in the expression levels of various aquaporin genes in response to waterlogging stress has been reported for several plant species including *Arabidopsis*, tobacco, soybean, and sorghum (Matsuo et al. 2012; Kadam et al. 2017; Tan and

Zwiasek 2019). As waterlogged roots encounter a general cell energy crisis, the expression of numerous genes is likely to be suppressed to conserve energy whereas hypoxia-inducible aquaporin genes are required for stress acclimation (Gibbs and Greenway 2003). The impact of aquaporins on K_r is mainly controlled by the gating mechanisms. Aquaporin gating, which controls the opening and closure of the water channels, is regulated by posttranslational modification such as phosphorylation, protonation, cytosolic Ca^{2+} and Rop-dependent H_2O_2 production (Baxter-Burrell et al. 2002). Waterlogged roots are likely to suffer from energy deprivation, which may be required to maintain the opening of aquaporins through phosphorylation (Kamaluddin and Zwiasek 2002). Aquaporin gating is also affected

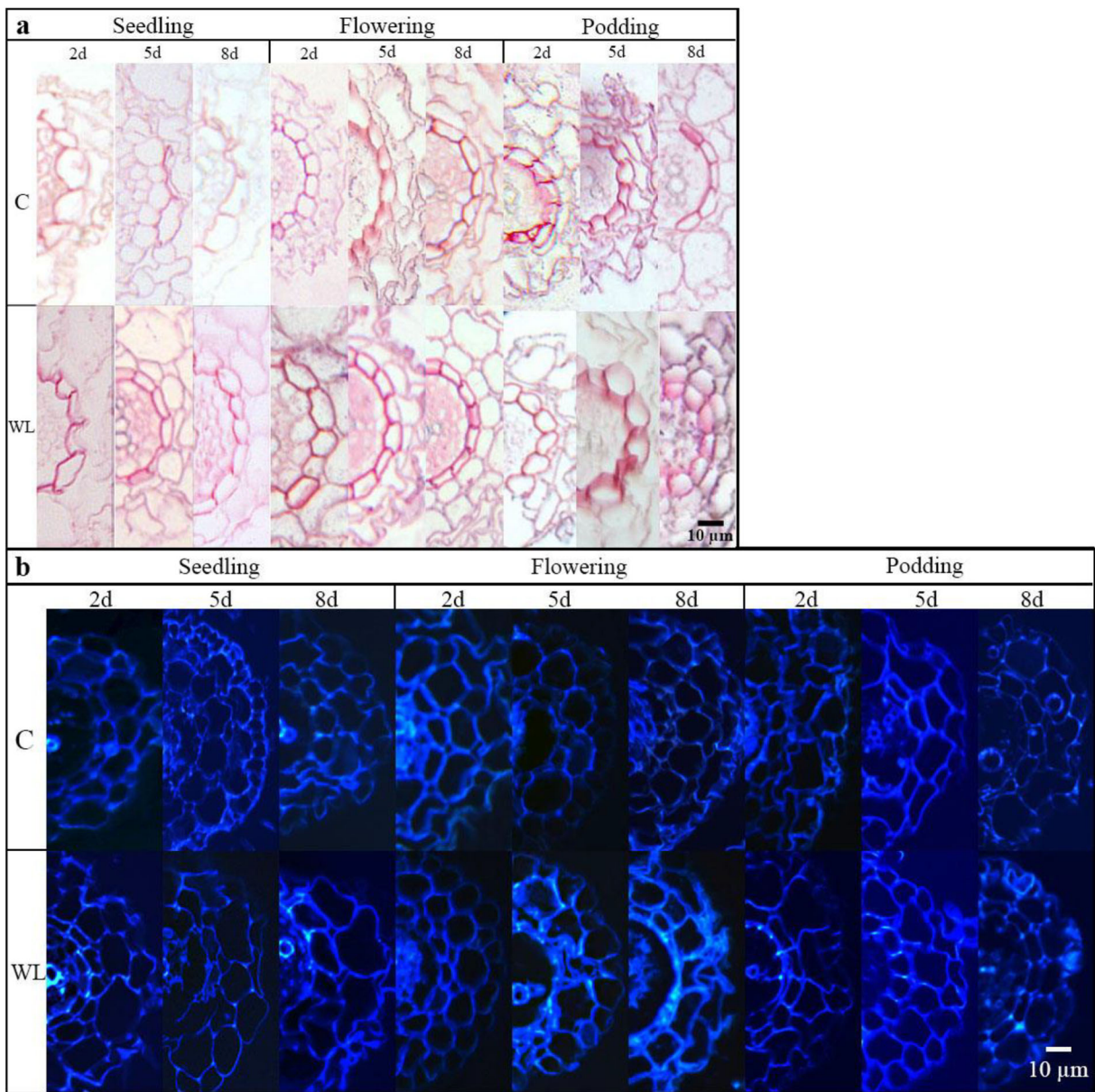


Fig. 6 Root cross sections from 3 to 5 cm from the end of root stained for suberin with Sudan 7B (a) and examined for lignin autofluorescence with blue light (b) in aerated control canola

plants (C) and in plants subjected to waterlogging (WL) for 2, 5, and 8 days at the different growth stages. Bars =10 µm

by the cytosol pH (Tournaire-Roux et al. 2003). Reduced cytosolic pH under hypoxic conditions lead to closure of aquaporin (Verdoucq et al. 2008) with K_r inhibition as a likely consequence. In our studies, the most upregulated genes by waterlogging included *BnPIP1;2*, *BnPIP1;3*, *BnPIP2;2* and *BnPIP2;7* and the function of these genes have not been revealed yet. In *Arabidopsis*, *AtPIP1;2* was demonstrated to

participate in CO_2 transport (Uehlein et al. 2012), while *NtPIP1;3* in tobacco is an effective oxygen transporter (Zwiazek et al. 2017). While the effectiveness of gas transport through PIP1s aquaporins has never been tested in canola, efficient CO_2 and O_2 transport in waterlogged roots would constitute an important hypoxia tolerance mechanism in plants (Boudichevskaia et al. 2015; Zwiazek et al. 2017). Since PIP2s are essential for

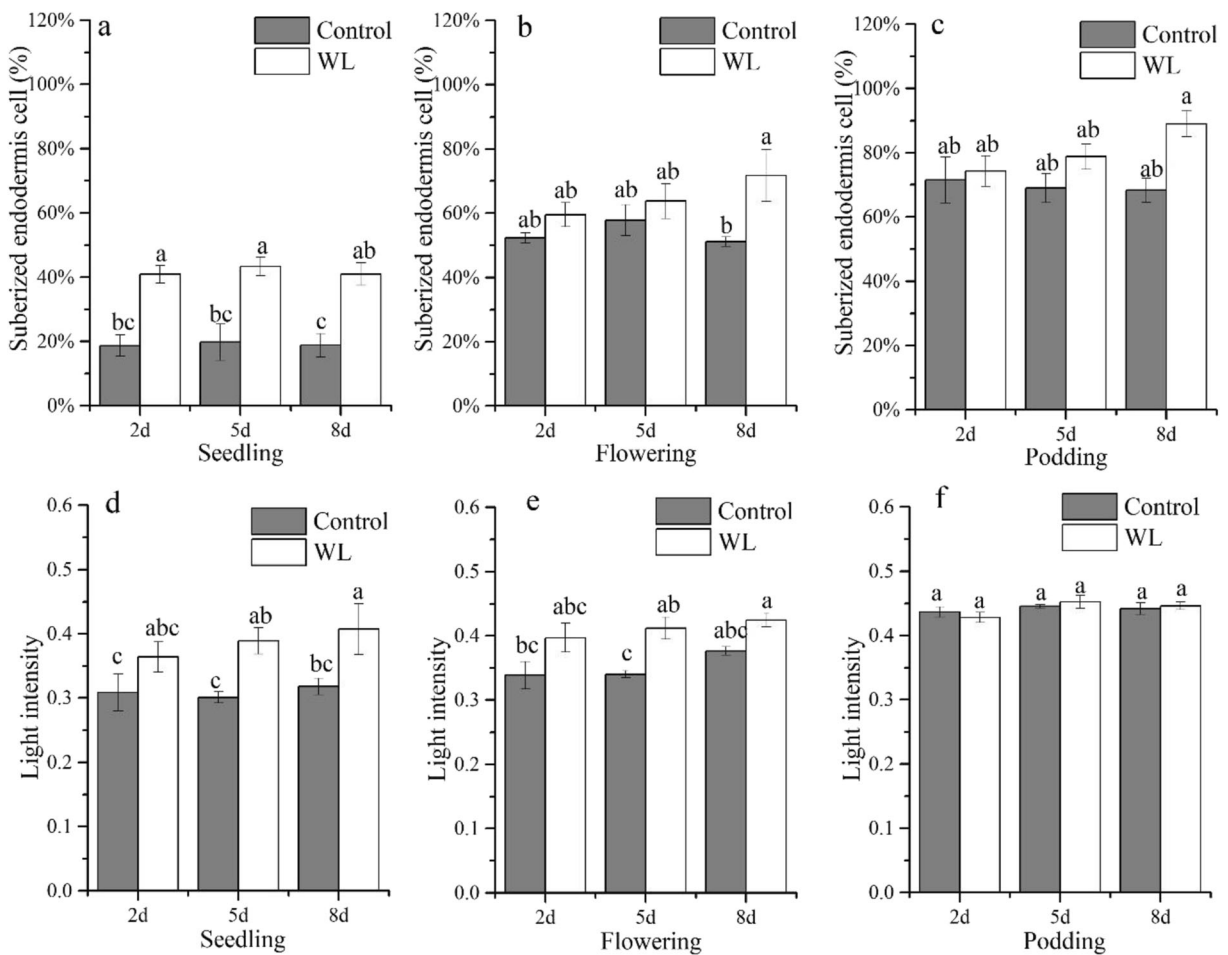


Fig. 7 Percentage of suberized endodermis cell in distal root segments and light intensity of lignified structures in distal root segments of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage (a, d).

F2, F5, F8 are 2, 5, and 8 days of waterlogging at the flowering stage (b, e). P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage (c, f). Means \pm SE are shown ($n=5$). Different letters above the bars indicate a difference determined by one-way ANOVA ($P \leq 0.05$; Tukey's test)

water transport, BnPIP2;2, BnPIP2;7 might be critical for regulating water relations in waterlogged roots (Ding et al. 2019) and their water transport properties and regulation mechanisms in canola should be further examined in the future.

To examine the possible reasons for the decrease in apoplastic transport, we compared the deposition of suberin and lignin in aerated and waterlogged roots. Waterlogging for 2 days enhanced the accumulation of suberin at the seedling stage. However, it took 5 days of waterlogging to see marked lignification of roots at the seedling stage. The presence of suberin and lignin restricts the root radial water uptake by reducing the diameter of pores of apoplast

(Hose et al. 2001). Increased suberin and lignin levels in roots significantly reduced water and solute permeabilities in rice roots (Zimmermann et al. 2000). However, in our study, lignification of waterlogged roots in plants at the podding stage did not significantly increase and there was a marked decline of apoplastic water transport at that time, suggesting that suberin might be mainly responsible for this reduction. Suberin and lignin deposition was also reported to be enhanced by various other environmental stresses including salinity, drought and heavy metal pollution (Byrt et al. 2018; Enstone et al. 2002). In waterlogged plants, suberin and lignin deposition in roots can help prevent pathogen infection,

reduce radial oxygen loss, as well as excessive loss of water and solutes (Kotula et al. 2009; Ranathunge et al. 2011; Kotula et al. 2014).

Conclusions

We observed dynamic changes in apoplastic and cell-to-cell contributions to root water transport following waterlogging at different growth stages in canola. The apoplastic transport declined with increased waterlogging duration and was accompanied by a deposition of suberin in waterlogged roots and by an increase in the proportion of the aquaporin-mediated transport. Several aquaporin genes including *BnPIP1;2*, *BnPIP1;3*, *BnPIP2;2* and *BnPIP2;7* were upregulated. Furthermore, waterlogging triggered significant declines in K_r , P_n and E within 5 days at the seedling growth stage, which was the most sensitive growth stage to waterlogging. The evidence points to the importance of maintaining functional aquaporins in waterlogged roots as a factor contributing to plant survival of waterlogging.

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