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Moderate drought stress increases resistance of *Brassica napus* to subsequent infection by *Leptosphaeria maculans*

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Abstract

Plants have developed adaptive strategies to cope with environmental stresses, but mechanisms effective under one stress may be counterproductive under others. This study investigates the effect of moderate drought stress pretreatment on the resistance of *Brassica napus* to *Leptosphaeria maculans*, the pathogen causing blackleg disease. *B. napus* plants were exposed to varying durations of drought stress, followed by a 24-h recovery period before inoculation with *L. maculans*. The results demonstrate a priming effect of the drought pretreatment, with a reduction in necrotic lesions in cotyledons compared to non-stressed controls. The most pronounced effect was observed in plants that underwent a 68-h drought pretreatment, resulting in a 45% reduction in disease symptoms. The transcriptions of 17 genes involved in *B. napus* defence against pathogen infection and drought stress were monitored. This revealed the involvement of the salicylic acid signaling pathway, indicated by increased expression of *PR1* and *PR2* marker genes. Additionally, drought stress marker genes were upregulated. These findings provide insight into the mechanisms of plant adaptation to combined biotic and abiotic stresses, which is essential for sustainable agriculture in a changing environment.

Keywords: biotic stress, *Brassica napus*, defence genes, drought stress, *Leptosphaeria maculans.*

Introduction

Plants are sedentary organisms that must adapt to a dynamic environment, which includes both biotic and abiotic stresses. Over the last century, climate change has become a significant threat to agriculture (Raza et al., 2019). Given the many variables - plants, pathogens, and environment, predicting how climate change might affect plant disease outcomes is rather difficult. Climate influences the fitness and pathogenicity of microorganisms, as well as their distribution and abundance (including geographic range and niche preference), and it shapes the co-evolutionary processes between plants and microorganisms, as well as the biology of plant hosts and vectors of pathogens. Climate change can also indirectly affect plant-pathogen interactions by altering the biochemistry and physiology

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Abbreviations: ABA - abscisic acid; ACS - 1-aminocyclopropane-1-carboxylate synthase; AOS - allene oxide synthase; CAT - catalase; CIPK6 - CBL-interacting protein kinase 6; dpi - day post inoculation; DW - dry weight; ET - ethylene; FW - fresh weight; HSF - heat stress transcription factor; ICS1 - isochorismate synthase 1; JA - jasmonic acid; LEA - late embryogenesis abundant; NCED - 9-cis-epoxycarotenoid dioxygenase; PAL - phenylalanine ammonia-lyase; PAMP - Pathogen-associated molecular pattern; PDF1.2 - plant defensin 1.2; PR - pathogenesis-related; RD - responsive to desiccation; ROS - reactive oxygen species; SA - salicylic acid; TF - transcription factor; VSP - vegetative storage protein; WRKY - pathogen-induced transcription factor; βCHI - β-chitinase.
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of the plant host and/or pathogen (Priya et al., 2023; Singh et al., 2023).

One of the key environmental elements influencing the performance and geographical distribution of different plant species is the water availability (Nawaz et al., 2023). Water deficiency causes a variety of responses in plants, including changes in morphology, physiology, and general metabolism. A lack of water can slow down the germination of seeds and the growth of young plants. Drought and pathogen infections can be mutually reinforcing (Kapoor et al., 2020; Ahluwalia et al., 2021). Plants have developed complex response systems to resist, reduce, or recover from various abiotic stresses (Wang et al., 2021). In the conditions of mild drought stress, a defence against pathogen infection overlaps with drought adaptation, sharing some signalling pathways. In the case of severe drought stress, in which the cells fail to maintain the integrity and some nutrients start leaking into the apoplast, plant adaptation based on signalling might not be sufficient, so disease symptoms might appear to be even stronger. Depending on the plant and pathogen, the combination of stresses can lead either to increased susceptibility or resistance (Kapoor et al., 2020; Ahluwalia et al., 2021; Priya et al., 2023). Drought stress is generally perceived by the roots as a decrease in water potential in the soil. The vascular system of plants connects roots and shoots and is crucial for combining stress information from the above- and below-ground parts of the plant. An abscisic acid (ABA)-driven hormonal signalling will be transduced to the aboveground parts, resulting in the closure of the stomata to reduce transpiration (Takahashi et al., 2020; Aslam et al., 2022). Hormone-like peptides integrate water deficit stress signals into long-distance organ-to-organ communication by acting as mobile molecules in the plant vasculature. For example, CLAVATA3/EMBRYO-SURROUNDING REGION-RELATED25 (CLE25) peptide regulates the ABA production and control stomatal closure during root-to-shoot signalling under the drought stress (Takahashi et al., 2020). Other important players in abiotic and biotic stress responses are transcription factors (TFs) of the MYB family. They modulate the expression of biosynthesis genes of metabolites including flavonoids, wax, and cutin in response to drought stress. Moreover, these TF families play a pivotal role in the stomatal movement through ABA signalling (Wang et al., 2021). WRKY TFs family has also a pivotal role in response to abiotic stresses. WRKY40 actively participates in plant responses to ABA and abiotic stress (Chen et al., 2020).

Abiotic stressors usually reduce susceptibility to biotrophic pathogens, but this effect is often reversed in hemibiotrophs and necrotrophs. A variety of abiotic stressors also modify plant transcriptome responses to biotic pathogens and increase their vulnerability to infection (Rivero et al., 2022). In a report comparing drought-stressed chickpea plants infected with *Macrophomina phaseolina* (the cause of dry root rot) or *Fusarium solani* (the cause of black root rot) to their corresponding single stress-treated controls, significant reductions in biomass and yield were observed. In parallel, a decrease in the number of lateral roots, root length density, and total root volume was observed (Sinha et al., 2019). Another study revealed that drought-stressed wheat plants infected with *Puccinia triticina* (the leaf rust causative agent) had a lower root dry weight, shorter root and shoot length, and a lower number of lateral roots (Naz et al., 2021).

Phytohormones are essential players in plant response to both biotic and abiotic stimuli. Among all hormones, the major role in stress responses is played by salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and ABA (Ciura and Kruk, 2018; Dubois et al., 2018; Li et al., 2019; Iqbal et al., 2022; Son and Park, 2022). ABA is a major regulator of stomatal closure, whether in response to drought (Takahashi et al., 2020), overflooding (Zhao et al., 2021), or pathogen penetration attempt through stomata (Melotto et al., 2006; Zhang et al., 2009). SA is a mediator and trigger of defence signalling at the proximal and distal infected tissues (Ali et al., 2018), and is also involved in stomatal closure regulation (Kalachova et al., 2013; Prodhan et al., 2018). SA accumulation is mostly associated with defence against biotrophic pathogens. JA and ET, on the other hand, are involved in the defence against necrotrophs or hemibiotrophs. JA participates in physiological (*e.g.*, regulation of stomatal movement) and molecular responses (including the interactions with TFs and other phytohormones) under abiotic stresses (Wang et al., 2020). ET is also involved in the regulation of the stomatal closure under drought stress. ET and JA cross-talk under several abiotic stresses has been reported that could be antagonistic or synergistic (Pérez-Llorca et al., 2023). Another important component of defence response is the formation of reactive oxygen species (ROS). ROS are produced by NADPH-oxidases and their signalling is considered as an early signal in plant defence response which occurs after minutes after PAMP recognition by cell surface receptors (Chapman et al., 2019; Son and Park, 2022). ROS accumulation can also be the result of decrease in activity of enzymatic ROS scavengers such as catalases and peroxidases, and they can be detected hours after infection by fungal pathogens (Nováková et al., 2014; La et al., 2019).

Brassica napus is cultivated in various regions in the world such as Australia, Europe, Canada, and northern China. Oilseed rape is adapted to diverse climatic conditions and contains three main ecotypes: spring, semi-winter, and winter type. The ecotypes differ by the vernalisation requirements for floral initiation (Khanzada et al., 2020). Hence, drought stress is the main stress to this crop due to its diverse cultivation areas (arid and semiarid). The devastating effect of water deficiency on *B. napus* plants results not only in disturbing essential physiological processes such as photosynthesis, and osmotic protection, but also in stunted growth and reduced oil content (Saeed et al., 2016; Chikkaputtaiah et al., 2017). Response of *B. napus* plants to drought stress varies according to the stress severity, cultivar, and phenological stages. Various effects of the drought as stomatal closure, osmotic adjustment, cell homeostasis, reduction of leaf expansion, and activation of enzymatic response have

been reported in *B. napus* (Zhu et al., 2017; Shawon et al., 2020; Ayyaz et al., 2021). Several studies have also provided abundant demonstrations concerning the impact of drought stress on plant biomass (shoot and root fresh and dry biomass) in other *Brassicaceae* species (La et al., 2019; Dai et al., 2020).

One of the most important fungal pathogens of *B. napus* is *Leptosphaeria maculans* (synonym *Plenodomus lingam*, class *Dothideomycetes*) (van de Wouw and Howlett, 2020), a causal agent of phoma stem canker disease, also known as "blackleg", spread across the world (Rouxel and Balesdent, 2005; van de Wouw et al., 2024). The pathogen infects plants in the early stages of their development by airborne ascospores that land on cotyledons or the first true leaves, and later by asexual conidia that are transmitted by water splashes within a plant or to neighbouring plants. Infection begins after germination of the spores and penetration of the pathogen into the cotyledon or leaf tissue. The first life stage of the fungus is biotrophic, when it colonises mesophyll tissue, followed by a necrotrophic life stage characterised by necrotic lesions and pycnidia formation. After this short period, the fungus spreads asymptomatically within the plant body for several months and, at the end of the season, forms the most serious symptom of this disease, the stem canker at the base of a stem, which blocks the transport of water and nutrients, resulting in premature ripening and loss of yield (Hammond and Lewis 1987). The key to resistance to this disease is to stop the pathogen at the initial stage before it enters the vascular tissues, so the role of abiotic factors influencing this stage is of great importance.

Our study investigates the effect of pretreatment of plants by drought on the infection process. The aim was to investigate whether a short period of drought stress can increase the resistance of plants to pathogen infection. For this purpose, *B. napus* seedlings were exposed to drought stress and inoculated with *L. maculans* after a short recovery period. The hypothesis that drought can lead to increased resistance or susceptibility to infection was tested, and possible mechanisms involved were discussed. To the best of our knowledge, this is the first study on the role of drought in this pathosystem.

Materials and methods

Plant and pathogen cultivation: Plants of *Brassica napus* L. (oilseed rape) cvs. Columbus and Eurol were grown hydroponically in perlite with Steiner's (Steiner 1984) cultivation medium under defined conditions (14-h photoperiod, temperature of 21°C, photon flux density of 150 µmol m^{-2} s^{-1}). Cotyledons were used in all experiments.

The fungus *Leptosphaeria maculans* (synonym *Plenodomus lingam*), isolate JN2 (Balesdent et al., 2005) was cultivated on V8 solidified medium (20% V8 vegetable juice, *Campbell Soup Company*, Camden, NJ, USA, 3 g L^{-1} $CaCO_3$, 15 g L^{-1} agar). Sporulation cultures and conidia suspension were prepared according to Šašek et al. (2012). After harvesting, the spores were diluted to 10^8 spore mL^{-1} in tap water and stored at -20°C for a maximum of 6 months.

Drought stress pretreatment and pathogen inoculation: The 11-d-old plants were subjected to drought stress for 68, 70, 73, and 75 h by leaving the plants without cultivation medium in dry trays. The wilting of the plants was observed (Fig. 1). Subsequently, the plants were irrigated with cultivation medium. After 24 h, the plants were completely recovered. The control plants were kept well-watered. Then, the plants were inoculated by the infiltration of conidial suspension of *L. maculans* into cotyledons and the development of the disease symptoms was monitored after 14 d. For all following experiments, the 68-h drought treatment was chosen, which enabled reproducible lesion quantification by image analysis.

For gene transcription, samples were collected by pooling material from cut discs (6 mm diameter) of 6 cotyledons. For dry weight determination, 6 whole cotyledons from 6 plants per treatment were dried at 100°C until constant weight. Proportion of dry matter was calculated as the ratio of dry mass to fresh mass.

The plants of age 15 d were used for inoculation with *L. maculans* isolate JN2 (Jindřichová et al., 2018). Cotyledons were infiltrated by spore suspension (10^5 spore mL^{-1}) using needleless syringe until full cotyledon saturation (approximately 100 - 120 µl). In order to measure the disease progression, the cotyledons of inoculated plants were sampled at 14 dpi and scanned

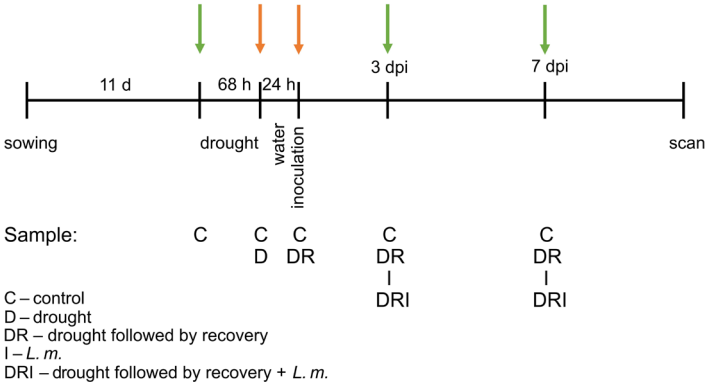

Fig. 1. The scheme of experiments. *Orange arrows* indicate sample collection for dry weight determination, *green* and *orange arrows* indicate sample collection for gene transcription.

by *Epson PERFECTION V700 photo* and then the lesion size was measured by *APS Assess 2.0* program using image analysis. Average lesion area of 12 control plants (24 cotyledons) was set as 100% and used for normalization.

Gene transcription analysis: Gene transcription in cotyledons was measured according to the method described previously (Šašek et al., 2012). Cotyledon discs, around 150 mg, were cut and immediately frozen in liquid nitrogen. The plant tissue was homogenized in tubes with 1 g of 1.3 mm silica beads and the total RNA was isolated using a *Spectrum Plant Total RNA* kit (*Sigma-Aldrich*, St. Louis, USA). The quantity and quality of RNA was evaluated on *Nanodrop 1000* (*Thermo Scientific*, Waltham, USA). Subsequently, 1 µg of RNA was treated with a DNAfree kit (*Ambion*, Waltham, USA) and converted to cDNA with a M-MLV RNase H-Point Mutant reverse transcriptase (*Promega*, Madison, USA) and oligo dT21 primer (*Metabion International AG*, Planegg, Germany). An equivalent of 6.25 ng RNA was loaded into a 10 µl qPCR reaction with the qPCR master mix (*LightCycler® 480 SYBR Green I Master* kit, Basel, Switzerland) carried out in a 96-well qPCR plate (*Axygen*, New York, USA) in *Light-Cycler 480* (*Roche*, Basel, Switzerland). The PCR conditions were as following: 95°C for 10 min, 45 cycles at 95°C for 10 s, 55°C for 20 s, and 72°C for 20 s; followed by a melting curve analysis. Threshold cycles and melting curves were calculated using *LightCycler* software *4.1* (*Roche*). The relative expression was calculated with an efficiency correction and normalization to the reference gene *Actin*. Used primer sequences are in Table 1 Suppl.

Statistical analysis: The experiments were carried out in three independent biological repeats (*i.e.*, three separate experiments carried out in different times), except for the gene transcription analyses, where two independent experiments were performed. Data from individual treatments of all replicates were averaged and analyzed using *t*-test and one-way *ANOVA* following Dunnett's multiple comparison test. Differences were considered to be significant at $P < 0.05$ (*) or $P < 0.01$ (**).

Results

The effect of drought stress followed by recovery on symptom development: To simulate environmental conditions of mild drought stress and recovery, we have exposed 11-d-old *B. napus* plants cv. Columbus to 68, 70, 73, and 75 h of drought stress (by non-watering) followed by the 24 h recovery phase prior to inoculation with spores of *L. maculans*. At 14 dpi, the progression of the disease was evaluated as the relative lesion area on infected cotyledons. Notably, short-term drought stress pretreatment made plants more resistant to fungal infection - relative lesion area was reduced by 50 and 52% at 68 and 70 h of drought stress, respectively, in comparison to control plants, well-watered throughout the experiment (Fig. 2). A similar result was obtained using *B. napus* cv. Eurol – 68-h drought stress pretreatment followed

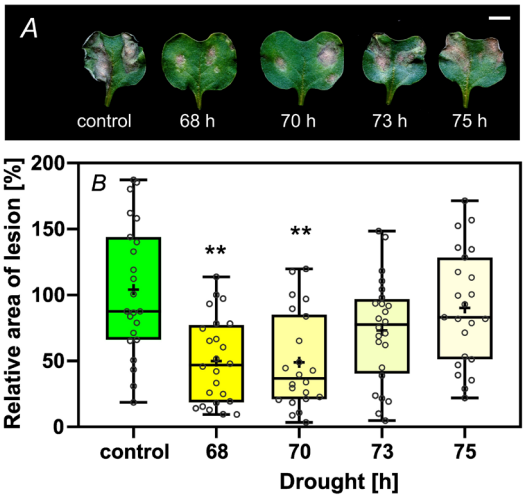

Fig. 2. Development of symptoms of *L. maculans* infection on *B. napus* cv. Columbus previously exposed to drought stress followed by recovery. Plants of *B. napus* (11-d-old) were exposed to the drought stress for 68, 70, 73, and 75 h followed by a 24-h recovery and then inoculated with *L. maculans* spore suspension. After 14 dpi, the disease symptoms of each treatment were evaluated as relative area of lesion when control treatment was set as 100%. *A* - representative photographs of *B. napus* cotyledons with disease symptoms, *bar* represents 1 cm. *B* - relative area of lesion [%]. *Box plots* cover interquartile range, *central line* represents the median, + *sign* corresponds to the average; ** indicate variants significantly different from control (one-way *ANOVA* with Dunnett's multiple comparison correction, $P < 0.01$, $n = 24$).

by the 24-h recovery phase caused a 45% reduction in the development of symptoms of *L. maculans* (Fig. 1 Suppl.). The priming effect of drought stress followed by recovery was not significant upon longer stress exposure (73 and 75 h), so for the following experiments we focused on the setup of 68-h drought pretreatment and *B. napus* cv. Columbus.

The effect of drought stress on fresh and dry weights of *B. napus* cotyledons: At the end of the drought stress exposure, mild turgor loss was observed in all drought-exposed plants. After re-watering (recovery phase), plants gradually recovered and at the end of 24-h recovery phase no visual differences were observed between pretreated and control plants. To better characterize the turgor dynamics upon mild drought stress prior to inoculation, we measured the fresh and dry weight changes.

The fresh (FW) and dry (DW) weight of cotyledons of drought-stressed plants of *B. napus* was measured at the endpoint of the drought stress (after 68 h of drought) and immediately after the recovery phase (Fig. 3). DW did not differ between drought-stressed and control plants, neither after drought nor after recovery period (Fig. 3*A*). In contrast, FW of drought-stressed cotyledons after drought was reduced by 28% compared to the control. This corresponded to the visually observed loss of turgor. After the recovery phase, the turgor was restored, but FW of the drought stressed plants was still 30% lower

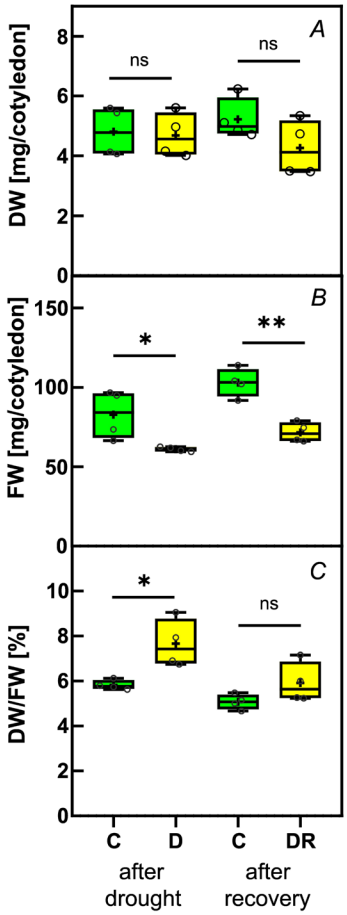

Fig. 3. Determination of dry weight (*A*), fresh weight (*B*), and their ratio (*C*) after drought stress (68 h) and recovery phase (24 h) in 11-day-old cotyledons of *B. napus*. C is control treatment, D is drought treatment, DR is drought treatment followed by recovery. *Box plots* represent the result of 4 values of each treatment and statistical analysis was done by *t*-test * $P < 0.05$, ** $P < 0.01$; ns - non-significant.

in comparison to the control plants (Fig. 3*B*). The DW/FW ratio was 30% higher in the drought-stressed plants at the endpoint of the treatment; and no statistically significant difference was observed after recovery, indicating restoration of turgor (Fig. 3*C*).

The effect of drought stress and *Leptosphaeria maculans* infection on stress-related transcriptome profile: To describe the effect of drought, fungal infection, and the stress combination on transcriptional landscape, we chose, on the base of our previous research and other relevant published data, a set of marker genes associated with particular signalling pathways. The relative transcriptions of 17 genes involved in *B. napus* defence responses (phytohormonal pathway and drought marker genes) were analyzed. Some of these genes were associated with salicylic acid (SA) biosynthesis, *e.g.*, isochorismate synthase 1 (*ICS1*) and phenylalanine ammonia-lyase (PAL); others were SA-responsive genes (also involved in pathogen response): pathogenesis-related gene 1 and 2 (*PR1* and *2*); the ethylene pathway marker genes, *e.g.*, biosynthetic gene ACC synthase (*ACS2*); responsive marker genes of the ethylene and jasmonic acid pathways β-chitinase (*βCHI*), plant defensin 1.2 (*PDF1.2*); jasmonic acid pathway marker genes, vegetative storage protein (VSP responsive gene), allene oxide synthase (*AOS*, biosynthetic gene) (Přerovská et al., 2022); abscisic acid (ABA) pathway marker genes: transcription factor responsive to desiccation 26 (*RD26*, responsive gene), and 9-cis-epoxycarotenoid dioxygenase 3 (*NCED3*, biosynthetic gene, Šašek et al., 2012); and drought stress marker genes: catalase (*CAT1*, Raza et al., 2021), late embryogenesis abundant (*LEA1*), heat stress transcription factor (*HSF22*, Zhu et al., 2017), CBL-interacting protein kinase 6 (*CIPK6*, Chen et al., 2012), MYB transcription factor (*MYB*, An et al., 2015), pathogen-induced transcription factor (*WRKY40*, Liu et al., 2015).

As expected, the transcription of drought maker genes and ABA-pathway marker genes was affected by 68-h drought stress (Fig. 4). The genes *RD26*, *NCED3*, *LEA1*, *HSF22*, *CIPK6*, and *MYB* showed a 3.6-fold, 3.7-fold, 86-fold, 4.2-fold, 5.3-fold, and 5.4-fold higher transcription, respectively, compared to the control treatment, and transcription of *WRKY40* was 2.5-fold decreased. After the recovery period (24 h after watering) transcriptions of *NCED3*, *LEA1*, *HSF22*, *CIPK6* returned to the control

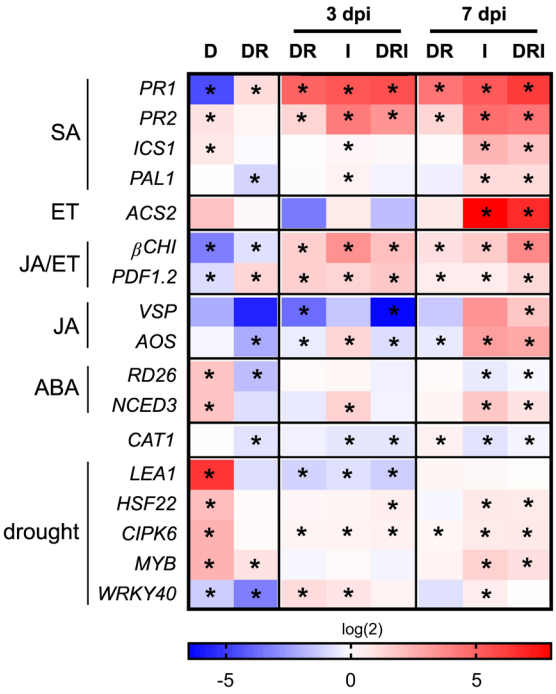

Fig. 4. Impact of drought stress, recovery, and *L. maculans* inoculation on transcription of stress marker genes in *B. napus* cotyledons. Plants were exposed to drought stress for 68 h (D), then watered (DR), and after 24 h of recovery period inoculated by infiltration of *L. maculans* spores (I). Samples were collected at the end of drought stress exposure, after recovery, 3 dpi and 7 dpi after inoculation (DRI). Heatmap represents relative transcription of genes of particular signalling pathways, log2 fold change. *Asterisks* indicate variants that significantly differ from the untreated control, $P < 0.05$, unpaired *t*-test, $n = 4$.

levels, while transcription of *MYB* remained 2-fold higher compared to the control and the transcription of *RD26* and *WRKY40* were 3.3-fold and 10-fold decreased. The defence marker genes were also affected by drought stress, especially those connected with the SA and JA/ET pathways. The transcription of *PR2* and *ICS1* increased after drought. On the other hand, the transcription of *PR1*, *βCHI*, and *PDF1.2* decreased 16-fold, 7-fold, and 2-fold after drought. After the recovery period, the transcription of *PR2* and *ISC1* returned to the control level, while transcription of *PR1* and *PDF1.2* was increased 2.2-fold and 2.5-fold, respectively; and the transcription of *PAL1*, *βCHI*, and *AOS* felt 2.2-fold, 1.7-fold, and 4.3-fold below the control level. After recovery and during further cultivation, *LEA1*, *CIPK6*, *WRKY40*, and *CAT1* transcription still differed between control and drought-stressed plants. After 3 d after drought followed by recovery (corresponding to 3 dpi point), *WRKY40* and *CIPK1* transcription was 2.2-fold and 1.4-fold increased, and *LEA1* 2.2-fold decreased in comparison to the control; after 7 d after drought followed by recovery (7 dpi point) only *CAT1* and *CIPK6* transcription was increased (1.4-fold, 1.3-fold). Drought stress followed by recovery also modified the transcription of SA-, JA-, and ET-associated defence genes: transcription of *PR1*, *PR2*, *βCHI*, and *PDF1.2* was elevated along the whole experiment, transcription of *VSP* was reduced after 4 d of recovery (3 dpi), and *AOS* was reduced at both time points.

Infection with *L. maculans* itself caused activation of the SA pathway (increase in transcription of biosynthetic and responsive genes) at 3 dpi and activation of the ET pathway (increase in transcription of biosynthetic gene) at 7 dpi; transcription of JA- and ET-responsive genes was also increased at 3 and 7 dpi. Notably, transcription of some drought marker genes was decreased in infected plants, *CAT1* at 3 and 7 dpi, and *LEA1* at 3 dpi. On the contrary, transcription of *HSF22*, *CIPK6*, *MYB*, and *WRKY40* was elevated in the infected plants, *CIPK6* and *WRKY40* in both time points and *HSF22* and *MYB* at 7 dpi.

The combination of drought followed by recovery and *L. maculans* infection induced the same plant defence signalling pathways as *L. maculans* infection itself - activation of SA and ET pathways (Fig. 4). When comparing plants exposed to double stress (drought pretreatment followed by recovery and inoculation) to inoculated-only (Fig. 5), *PR1* transcription was 1.7-fold higher at 7 dpi in combined stress condition. On the other hand, the transcription of *PAL1* in the combined stress condition at 3 dpi was 1.6-fold lower than that of the infection-only. Under combined drought followed by recovery and infection, transcription of *VSP* and *AOS* was suppressed 20-fold and 4.5-fold at 3 dpi, *βCHI* transcription was 2.7-fold lower at 3 dpi but 4-fold higher at 7 dpi, and *PDF1.2* transcription was 1.6-fold elevated at 7 dpi in comparison to non-stressed *L. maculans* inoculated plants. Moreover, activation of the ABA pathway under combined stress was lower than in response to single infection with *L. maculans* (*RD26* 1.5-fold, *NCED3* 3.3-fold), except at 7 dpi when transcription of *RD26* was 1.2-fold higher. Taken together, considering the known antagonism

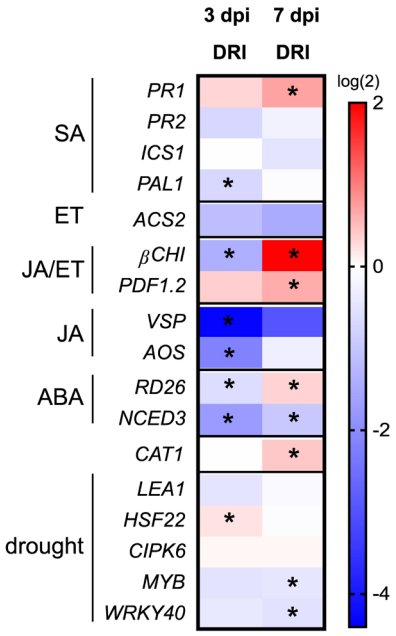

Fig. 5. The effect of drought stress followed by recovery on the transcriptional response to *L. maculans* infection in *B. napus* cotyledons related to non-stressed inoculated plants. Relative transcription of defence and drought stress marker genes in *L. maculans* infected plants subjected to drought stress followed by recovery period before inoculation in comparison to non-stressed inoculated plants, log2 fold change. *Asterisks* indicate variants that significantly differ from the untreated control, $P < 0.05$, unpaired *t*-test, $n = 4$.

between the SA and JA pathways, the downregulation of the JA pathway in drought-stressed and recovered plants may lead to an elevation of the SA pathway. A similar effect can be observed in the ABA pathway under stress conditions (Fig. 5). Drought stress marker genes (*CAT1*, *HSF22*, *MYB*, and *WRKY40*) had different transcriptional profile in combined stressed cotyledons and inoculated cotyledons. *HSF22* was increased under both types of stress at 3 dpi (1.2-fold), and both *MYB* and *WRKY40* were 1.4-fold decreased under combined stress in comparison to single *L. maculans* infection at 7 dpi.

Discussion

Climate change subjects plants to two major abiotic stresses in field conditions: water insufficiency and elevated temperatures (Chen et al., 2020; Priya et al., 2023). Numerous studies on plant responses to combined abiotic and biotic stresses indicate that plants utilize distinct mechanisms depending on the specific stress combinations they encounter (Ramegowda and Senthil-Kumar, 2015).

For instance, drought-stressed tomato plants have been shown to exhibit increased resistance to *Botrytis cinerea* infection (Achuo et al., 2006). Conversely, virulent and avirulent strains of *Pseudomonas syringae* pv. *tomato* proliferated more effectively in heat-stressed *Arabidopsis thaliana* plants (Wang et al., 2009).

Several studies on the simultaneous effects of *Dothideomycete*-induced diseases and drought stress have revealed the alterations of host-pathogen interactions. In a comparison between well irrigated and long-term drought-stressed barley (*Hordeum vulgare*) plants in the field, drought stress was found to enhance resistance of plants to *Ramularia* leaf spot caused by *Ramularia collo-cygni* (Hoheneder et al., 2021). Another study demonstrated that overexpression of the barley stress-responsive NAC transcription factor (*SNAC1*) reduces the symptoms of *Ramularia* leaf spot and fungal colonization even in the absence of stress. The authors identified *SNAC1* as a key player in abiotic stress tolerance, inhibition of senescence, and as a mediator of resistance to *R. collo-cygni* (McGrann et al., 2015a,b). In contrast, drought stress increased the severity and incidence of dry root rot caused by *Macrophomina phaseolina* in chickpea. The increased fungal colonization was accompanied by decreased transcription of several defence genes (Irulappan et al., 2022). The combination of drought and *Pseudomonas syringae* pv. *tomato* DC3000 infection illustrated the reduction of pathogen multiplication by drought stress (Gupta et al., 2016). However, another study revealed that plants of *Arabidopsis thaliana* under moderate drought stress were more vulnerable to *Pseudomonas syringae* pv. *tomato* DC3000 (Choudhary and Senthil-Kumar, 2022). Recently, a valuable resource, the plant stress informatics hub (*SCIPDb*), has emerged. This database offers data on the morpho-physio-biochemical (phenome) and molecular (transcriptome and metabolome) responses of plants to various stress combinations. It includes data from 123 stress combinations (Priya et al., 2023). However, there is a lack of data concerning drought pretreatment followed by fungal infection.

As can be observed from the aforementioned data, the majority of these studies concern a combination of drought and pathogen infection. The impact of drought pretreatment on plants, resulting in a priming effect, is limited. For instance, drought-primed eucalyptus plants demonstrated reduced susceptibility to a fungal pathogen, *Neofusicoccum eucalyptorum* (Barradas et al., 2018). Similarly, drought pretreatment resulted in tolerance to the necrotrophic fungus *Sclerotinia sclerotiorum* and the hemi-biotrophic bacterial pathogen *Pseudomonas syringae* pv. *tabaci* in *Nicotiana benthamiana* (Ramegowda et al., 2013). Additionally, mild water stress significantly delayed the onset of symptoms in primed avocado plants infected with *Rosellinia necatrix* (Martínez-Ferri et al., 2019).

The goal of our study was to investigate the effect of priming the *B. napus* plants by drought on symptom development of *L. maculans*. The study of this pathosystem makes sense for several reasons: *B. napus* is a very important oilseed crop worldwide, and *L. maculans* is a serious pathogen with a hemibiotrophic lifestyle that can be affected by drought stress. Under natural conditions, cotyledons or the first true leaves are commonly infected with spores. Thus, we used cotyledons for experiments, which, unlike true leaves, have a stronger immune response and the extent of infection can be easily quantified by the extent of necrotic lesions. In our experimental setup, drought stress was relatively mild, consisting of non-watering of hydroponically grown plants for 68 - 75 h followed by watering and 24-h recovery prior to inoculation. Several studies have reported the effect of drought stress on plant biomass such as shoot and root fresh and dry weight in *Brassicaceae* species including *B. napus* (Farhat et al., 2019; Dai et al., 2020), as well as reduction in relative water content, osmotic potential, and potassium content (Khan et al., 2010). Notably, while drought did not affect the dry weight of cotyledons, fresh weight decreased after stress and remained lower than that of the control even after recovery, indicating deeper physiological changes associated with adaptation to stress.

We monitored the transcriptional profiles of drought stress-associated genes through the experiment. Initially, we assessed the transcription of known drought stress markers. Elevated transcription of the *LEA1* gene in non-inoculated and drought-stressed plants in our experiments highlights its importance in drought adaptation in *B. napus*. Indeed, the *LEA1* gene was upregulated under drought and salt stresses in tomato (Cao and Li, 2015). In our experiments, transcription of three other drought stress genes *HSF22*, *CIPK6*, and *MYB* was slightly elevated after 68 h of the drought stress, while the transcription factor *WRKY40* was downregulated in the same plants. This aligns with previously reported results, where *WRKY40* was downregulated in *Arachis duranensis* under drought stress (Zhang et al., 2022), and the *MYB* gene from *Poncirus trifoliata* was upregulated upon dehydration (Sun et al., 2014). The accumulation of ABA and defence-related proteins confers drought tolerance in *B. napus* (Zhu et al., 2010). The ABA signalling pathway is conserved in *B. napus* (Zhu et al., 2016), and genes involved in ABA signalling pathway are typically upregulated under drought stress (Li et al., 2005; Zhu et al., 2010). Under drought stress, content of endogenous ABA elevated by about ten times compared to control plants (Huang et al., 2008). Indeed, we detected upregulation of the ABA biosynthetic gene *NCED3* and ABA-responsive gene *RD26* after drought stress. In contrast, we observed downregulation of JA biosynthetic marker gene (*AOS*) after stress and 24-h recovery in comparison to well-watered controls. *AOS* transcription was reported to be elevated in grapevine under drought stress (Haider et al., 2017). We also observed drought-induced downregulation of genes related to JA/ET signalling pathway, such as *βCHI* and *PDF1.2*. While *βCHI* remained low after recovery, *PDF1.2* exhibited the opposite trend, highlighting the complexity of this regulatory pathway. Drought stress and recovery also led to changes in the SA-related transcriptome, involving both biosynthetic (*ICS1*, *PAL*) and responsive genes (*PR1*, *PR2*). This highlights the crucial role of SA signalling in *B. napus* defence against *L. maculans* infection and adaptation to drought stress. Taken together, these results demonstrate that moderate drought stress modifies transcriptomic landscape, and the major defence-related pathways are significantly affected, which may be reflected in plant response to subsequent pathogen exposure.

After inoculation by *L. maculans* we observed smaller

lesion area in drought pre-stressed plants in comparison with non-drought-stressed plants. Among the tested conditions, only 68 and 70 h of drought pre-stress reduced lesion formation, illustrating the biologically relevant priming effect of moderate drought stress on the *B. napus* immune system. This effect is further strengthened by the decrease of *PR1* transcription by drought itself and its immediate increase upon recovery to the levels exceeding those in the well-watered plants. SA-dependent pathway plays an important role in the *B. napus* defence against *L. maculans* as seen by elevated transcription of SA-responsive genes *PR1* and *PR2* and SA biosynthetic genes (*ICS1* and *PAL1*) in all inoculated plants. This aligns with previous studies describing the activation of the SA signalling pathway under the interaction between *B. napus* and *L. maculans* (Šašek et al., 2012), and other reports demonstrating a positive relationship between increased expression of *PR1* and *PR2* genes and SA signalling pathway activation (Kunkel et al., 1993; Delauré et al., 2008).

Transcription analysis was performed at 3 dpi (early stage of infection by *L. maculans*) and 7 dpi (late stage of infection). Since *L. maculans* spores begin to germinate in the apoplast at 3 dpi, the cells are initially exposed to fungal PAMPs and metabolites. At 7 dpi, the first lesions start to develop at the infected leaves, indicating a transition between biotrophic and necrotrophic phases of *L. maculans* growth (Li et al., 2006). The defence against biotrophs relies heavily on SA-related pathways (*PR1*, *PR2*, *ICS1*), out of which we only detected *PR1* to be enhanced by drought pretreatment in comparison to inoculated well-watered plants, and only at 7 dpi. We observed upregulation of *ACS2* transcription at 7 dpi, confirming the active involvement of the ET pathway in the defence against *L. maculans* during the necrotrophic phase. The *ACS2* encodes an enzyme involved in the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ET and is responsive to wounding and osmotic stress (Denekamp and Smeekens, 2003). JA and ET signalling pathways often overlap in plant defence responses (Broekaert et al., 2006). In our study, an upregulation of the *βCHI* gene (JA/ET gene marker) was observed at both 3 dpi and 7 dpi. Other JA/ET and JA-responsive genes, *PDF1.2*, *VSP2*, and *AOS* were upregulated at 7 dpi by drought stress pretreatment in comparison to well-watered infected plants. Upregulation of JA/ET marker genes corresponded to published transcriptomics profile of the infected plants for *PDF1.2* (Becker et al., 2017). On the contrary, *AOS* decrease was previously reported in infected *B. napus* at 7 dpi (Becker et al., 2017). This discrepancy may be due to differences in experimental setup, particularly in plant cultivation conditions or type of inoculation method. However, it is known that ABA plays an important role in plant defence mechanisms by regulating the stomata, it is an important hormone for drought signalling and also for signalling during *L. maculans* infection (Šašek et al., 2012). Interestingly, under combined drought stress and *L. maculans* infection, synergism was not observed. At 3 dpi, *RD26* and *NCED3* transcription was lower in double stressed plants compared to only inoculated plants. At 7 dpi, the *RD26* transcription was higher in double-stressed plants but the transcription of biosynthetic gene *NCED3* was decreased. This finding suggests that ABA signalling is much more complex in drought-treated and inoculated plants. The enzyme catalase is actively involved in plant defence, development, and senescence by degradation of H_2O_2. The transcription of *CAT1* was shown to be increased under ABA treatment (Raza et. al., 2021). Surprisingly, we observed the downregulation of *CAT1* under drought stress, and also after inoculation *CAT1* was downregulated. This finding is inconsistent with an increase in catalase enzyme activity (Jindřichová *et al*., 2011), but transcription of *CAT1* may depend on the time of observation. The transcriptional profile in this study provides a comparative analysis of several marker genes in phytohormone signalling pathways and drought pathways in *B. napus*. Overall, these results open insight into the mechanisms of phytohormone signalling cross-talk under drought followed by pathogen infection.

Conclusion

With climate change leading to more frequent and severe droughts, it is critical to understand how water stress affects plant susceptibility to disease. Our study is a novel investigation of how pretreatment of *B. napus* plants with a drought stress affects the course of its fungal infections and provides new insights into the dynamics of plant diseases under environmental stresses.

Our results show that the pretreatment of *B. napus* by a mild drought stress can prime the plants to increase the resistance to fungal pathogen *L. maculans* in cotyledons, suggesting that environmental factors such as drought can influence both the severity and progression of plant diseases. To elucidate the mechanisms involved, we compared transcriptomic profiles at different stages of stress and disease development, focusing on marker genes of specific phytohormonal signalling pathways. The results suggest that the key to drought resistance may lie in ABA and JA/ET cross-talk with SA. There is evidence of an antagonistic relationship between SA and JA in defence responses, so it is possible that inhibition of JA and ABA signalling allowed SA-related immunity to "more efficiently" limit *L. maculans* proliferation. On the contrary, at the later stage of infection, when the fungus transits from the biotrophic to the necrotrophic phase and JA- and ET-based defences become important, transcription of JA-responsive genes was more strongly activated in drought-stressed plants. Our data demonstrate for the first time the role of drought priming in *B. napus* resistance against *L. maculans* infection and the associated phytohormonal interactions. Thus, our study emphasises the need for similar studies in other species of the *Brassicaceae* family, which includes many agriculturally important crops and would therefore be of great economic importance.

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