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Application of Barley *Tweaky Spike* Mutants for the Study of Effects of Plant Immunity-Related Substances

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Abstract: Barley developmental mutants *tweaky spike* (*tw*) with disturbed auxin pathways possess a unique feature of an increased level of mouldy germinating grains (MGG), which serves as a convenient model to investigate the effects of plant immunity-related substances. The effects of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D), auxin inhibitors, salicylic acid (SA), and *trans*-cinnamic acid (TCA) were studied using the *tw-WT* system in surface-sterilized and unsterilized germinating grains under high rates of natural infection. Significant differences among the allelic *tw* mutants were revealed at the natural MGG level and in response to 2,4-D, SA, and TCA. The most effective means against MGG were sterilization and TCA. 2,4-D inhibited root growth in *tw* and tw_2 mutants, occurring only in unsterilized and not sterilized germinating grains, while the opposite was observed for TCA and SA. The *tw* mutations influenced variations in the seed-borne fungal spectra, decreasing the frequency of *Bipolaris sorokiniana* and increasing *Fusarium* spp. Hypochlorite-based surface sterilization methods should be used with caution in studies where the action of exogenous 2,4-D will be analysed in germinating grains. Auxin pathway disturbances specific for pleiotropic *tw* mutants are generally restricted to organogenesis but not to germination events.

Keywords: barley; MGG assay; salicylic acid; *trans*-cinnamic acid; *tweaky* mutants; 2,4-D

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1. Introduction

Grain contamination with fungi and their produced mycotoxins is not only a problem for organic producers but also for conventional agriculture [1]. For barley, special attention is required for malt and beer contamination with mycotoxins and their proper control and avoidance [2].

A group of barley pleiotropic *tweaky spike* (*tw*)-type mutants (*tw*, tw_1, tw_2) possess a specific tweaked spike structure (Figure S1) and an irregular transformation of lodicules, which are grass-specific floral organs, intoreproductive or chimeric organs. They are also characterized by higher susceptibility to *Claviceps purpurea* and *Ustilago nuda*, and increased levels of germinating grain moulding [3,4]. The higher susceptibility of *tw*-type mutants to fungal pathogens may be explained by their polar spike architecture with the semi-sterile lower part of a spike and overdeveloped multi-rowed upper part, in which flowers with ectopically developed extra floral organs become partially opened during the grain development stage, making it more accessible to external fungal spores and rainwater [3]. Nevertheless, the increased level of mouldy germinating grains (MGG) is unique and deserves further study. Grain infection by fungal pathogens is among the main sources of plant infection [5,6], and grain-borne fungi cause significant grain yield and quality losses via toxin production due to the resulting decrease in the germination rate and the increased number of abnormal and affected seedlings [7]. Although the mechanisms leading to MGG of barley *tw*-type mutants are poorly studied, this phenomenon can still be employed as

a convenient and simple model to test the effects of plant immunity-related substances, making barley *tw*-type mutants a suitable genetic system for an MGG-based assay.

Our recent studies have related the *tw* phenotype to disrupted auxin physiology [8,9], providing a pretext to study the role of auxin in the MGG phenomenon. Depending on lifestyle, phytopathogens develop means for controlling plant growth by modulating endogenous auxin synthesis and signalling [10]. Moreover, endophytic auxin-producing fungi may improve plant growth, suppress seed-borne infections, and enhance resistance to abiotic stresses [11,12]. On the other hand, plants evolve mechanisms to repress auxin signalling during pathogen infection as a defence strategy [13,14]. The downregulation of auxin signalling is part of the pathogen-induced host immune response [15]. Mutations in genes responsible for auxin synthesis and signalling are most often used to study the role of auxin in response to pathogen infection. Through the use of these mutations, significant differences in plant auxin–pathogen relationships have been demonstrated for the infection stage [16], different species of the same fungal genus or the same fungus on different plants [17].

The present study aimed to relate the increased levels of MGG in *tw* mutants with disturbances in the auxin pathway and to employ MGG as an assay to test the effects of potential modifying factors against pathogen manifestation in germinating grains. The effects of grain sterilization and various treatments, including synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), auxin transport inhibitor 9-hydroxyfluorene-9-carboxylic acid (HFCA), antiauxin p-chlorophenoxyisobutyric acid (PCIB), phytohormone salicylic acid (SA), and its precursor *trans*-cinnamic acid (TCA), were studied on MGG and root growth inhibition, the most pronounced indicator of response to 2,4-D [18], using three allelic *tw*-type mutants (*tw*, tw_1, tw_2). In previous studies, the *tw*-type mutants were used to study the effects of the antioxidants ascorbic acid and glutathione [19] and SA [20] on MGG frequency and root growth, and the transgenerational transmission of the effects of SA treatment [21]. In the present study, the effects of exogenous 2,4-D, auxin inhibitors, SA, and TCA on MGG and root growth, were compared using a palette of various *tweaky*-type mutants and *WT*. The influence of *tw*-type mutations on the frequency of *Bipolaris* and *Fusarium*, two predominant genera of fungi found in the internal tissues of all tested barley genotypes, is also emphasized.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The plant material used for the study was propagated in an experimental field at the Botanical Garden of Vilnius University, using 2×2 m^2 plots per genotype, 600–800 plants per plot. All the plant material had been cultivated for many years without pesticide exposure to retain the natural diversity and frequency of plant pathogens.

Recessive allelic *tweaky spike*-type mutants, *tw*, tw_1 and tw_2, were induced by chemical mutagenesis using ethylene imine in the barley cv. Auksiniai II, which has the same genetic background, was used as the *Wild type* (*WT*) (Figure S1). During the early phase of stabilization (F_4-F_6), tw_1 and tw_2 mutants underwent a period of genetic instability, which led to complete or incomplete reversions to the *WT* phenotype (*tw* → *Tw*), resulting in a large collection of revertants. In the case of incomplete reversion, some revertants preserved several *tw*-specific features, such as higher protein content in grains and increased resistance to lodging [22]. In the present study, six revertants (N1, N13 and C1—from tw_1, N46, C6 and C7—from tw_2) were used for the additional comparison of internal grain fungi.

Two other *tweaky*-type mutants, *tweaky N18* (*twN18*) and *tweaky and missing kernels* (*twmk*), were also included in the analysis of 2,4-D effects on MGG and root growth. Both mutants were obtained from the USDA-ARS National Small Grains Collection (Aberdeen, ID, USA) and possess tweaked spikes, but their susceptibility to fungal infections is unknown. The complementation test revealed *twN18* and *twmk* mutants to belong to complementation groups other than the allelic *tw*-type mutants [23]. The grains used in the present study for the MGG analysis were selected from different years of growth, and

stored at +4 °C in the dark until the analysis was performed. The years of reproduction are indicated in the legends of Figures 3–5 and the footers of Tables S3–S5.

2.2. Mouldy Germinating Grain (MGG) Test

All manipulations were performed under sterile conditions. The seed material was divided into two groups: (1) nonsterilized, deionized water-imbibed grains to evaluate the natural infection level and (2) grains surface-sterilized with a water-diluted commercial bleach ACE solution (1:1, *v*/*v*) for 15 min and then washed with sterile water three times for 2 min. The grains were then soaked in solutions of synthetic auxin 2,4-D (50–800 mg L^{-1}), the auxin transport inhibitor 9-hydroxyfluorene-9-carboxylic acid (HFCA) or antiauxin p-chlorophenoxyisobutyric acid (PCIB) (200 µM), phytohormone SA or its precursor TCA (0.25–1.0 mM) for 12 h, rewashed with autoclaved water and placed in Petri dishes (ten grains per dish) with three layers of wet filter paper. The frequencies of MGG, germination rate, and root length were determined after five days of growing at 25 °C in the dark. Mouldy grains were scored and removed from the Petri dishes every day to prevent infection of the neighbouring grains. All the experiments were performed in three independent replicates, ten Petri dishes with 10 grains per dish (i.e., 100 grains in total) were used per treatment. For root length measurements, roots of 30 germinating grains were measured in total (i.e., 10 germinating grains per experimental replicate). The concentrations of the compounds were selected from small-scale experiments and our previous studies [8,9]. 2,4-D was manufactured by Alfa Aesar (Germany), and all the other compounds were obtained from Sigma-Aldrich (USA).

2.3. Identification of Internal Fungi in Barley Grains

The pure culture method was applied for the detection of fungi inside the seeds [24]. The grains were sterilized with 3% NaClO for 2 min and washed with sterile water three times for 2 min. The grains were then dried with sterile filter paper, placed on Petri dishes containing malt extract agar (MEA) medium (2%) and streptomycin (250 mg L^{-1}), and maintained at 24 °C for seven days in the dark. The fungi were identified according to their morphological and cultural characteristics as described by Mačkinaitė [25].

2.4. Investigation of Effects of Meal on B. sorokiniana Growth

To study the potential inhibitory effects of meal from *WT* or allelic mutants *tw*, tw_1, and tw_2 under the background of SA or TCA, pure cultures of *B. sorokiniana* were isolated from sterilized barley grains using a single spore technique and deposited in the collection of the Institute of Botany, Nature Research Centre, Vilnius, Lithuania. The isolate of *B. sorokiniana* was reidentified by a taxon-specific polymerase chain reaction. Briefly, genomic DNA was extracted from 50 mg of wet mycelium using Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corporation, Thorold, Canada) following the manufacturer's recommendations. Species-specific PCR was carried out according to [26] using the Green PCR Master Mix (Thermo Scientific™, USA) and species-specific primer pair (FW 5′GGTCCGAGACAACCAACAA3′ and RV 5′AAAGAAAGCGGTCGACGTAA3′). The 600 bp species-specific SCAR PCR product was electrophoresed in 1.2% agarose gel containing ethidium bromide (0.5 µg mL^{-1}) in 1× Tris–Borate–EDTA buffer.

Meals from *WT* and different *tw*-type mutants (60 g L^{-1}) and SA or TCA (0–1 mM) were added directly to the MEA medium before autoclaving. The diameter of *B. sorokiniana* colonies was measured after seven days of incubation at 24 °C in the dark.

2.5. Statistical Analysis

The results are representative of three independent experiments performed at least in triplicate and are shown as the mean values ± standard error (SE) of the mean in the tables and figures. The assumption of data normality was assessed through the Shapiro–Wilk test using Past (version 3.26) software. Since at least some of the data sets did not follow a normal distribution, a nonparametric Kruskal–Wallis rank-sum (omnibus) test was

performed using Statistica 64 (version 12.0) software. Dunn's test with Benjamini–Hochberg (FDR) adjustment was used as a *post hoc* test to examine the differences between the sterilized and unsterilized grains or multiple pairwise comparisons of different treatments and the control.

3. Results

3.1. The Action of 2,4-D, SA and TCA on MGG and Root Growth of Allelic Tweaky Spike Mutants

Naturally, grain sterilization before germination is an effective treatment against MGG. In most cases studied, the MGG level was significantly lower in surface-sterilized grains than in unsterilized grains under the same experimental conditions, and independent of the plant genotype and the studied 2,4-D concentrations (Figures 1a and 2a, Tables S1a and S2a).

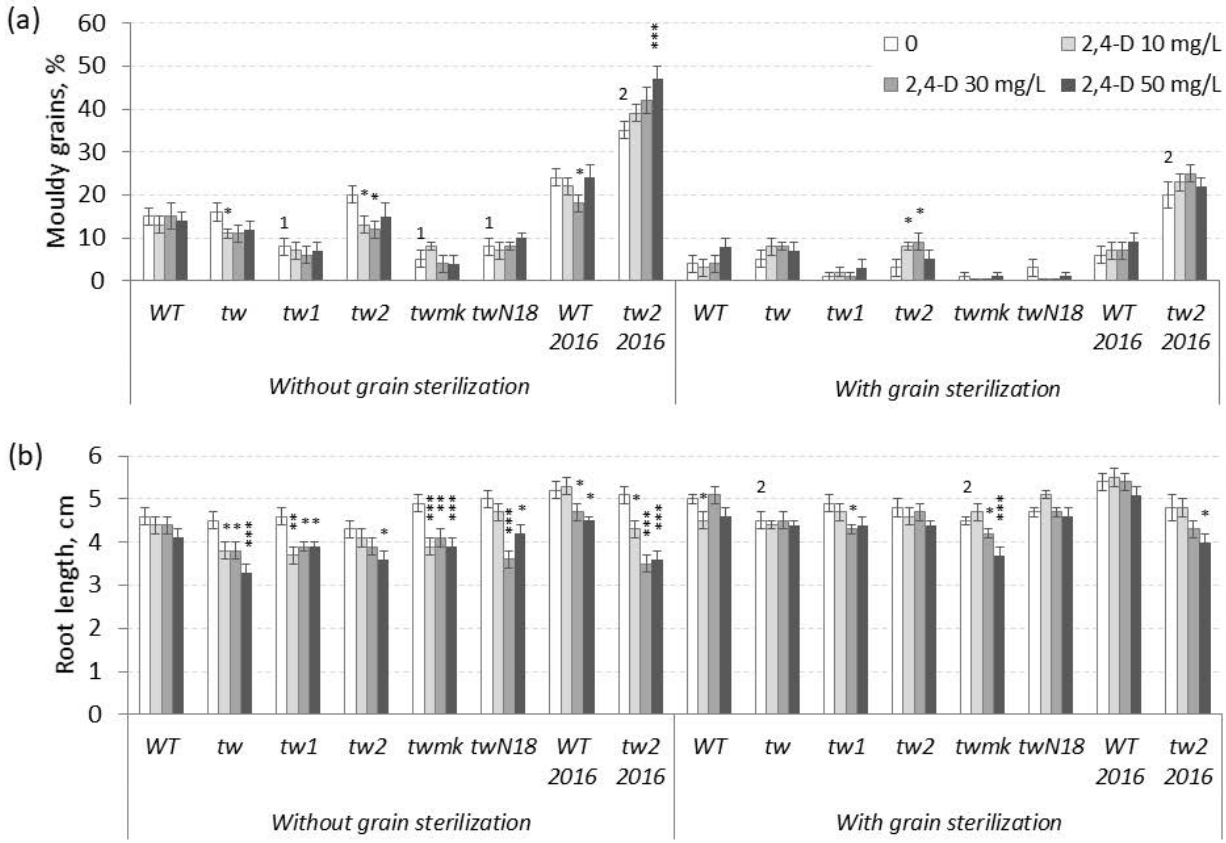

Figure 1. Effects of 2,4-D over a 10–50 mg L^{-1} range on barley *tweaky*-type mutants. (**a**) The frequency of mouldy grains (%) and (**b**) the root lengths (cm) of unsterilized and sterilized germinating grains measured after 5 days. For mouldy grains, n = 10 *Petri* dishes (100 grains); for root length n = 30; for tw_2, n = 15 *Petri* dishes (150 grains) and n = 60. *WT2016* and $tw_2 2016$, grain reproduction in 2016; other material, grain reproduction in 2013; the MGG assay was performed in 2017. The asterisks represent significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) between the control and the 2,4-D treatment. The numbers denote significant differences (^{1} $p < 0.05$; ^{2} $p < 0.01$) between the controls of the *tw*-type mutant and the *WT*.

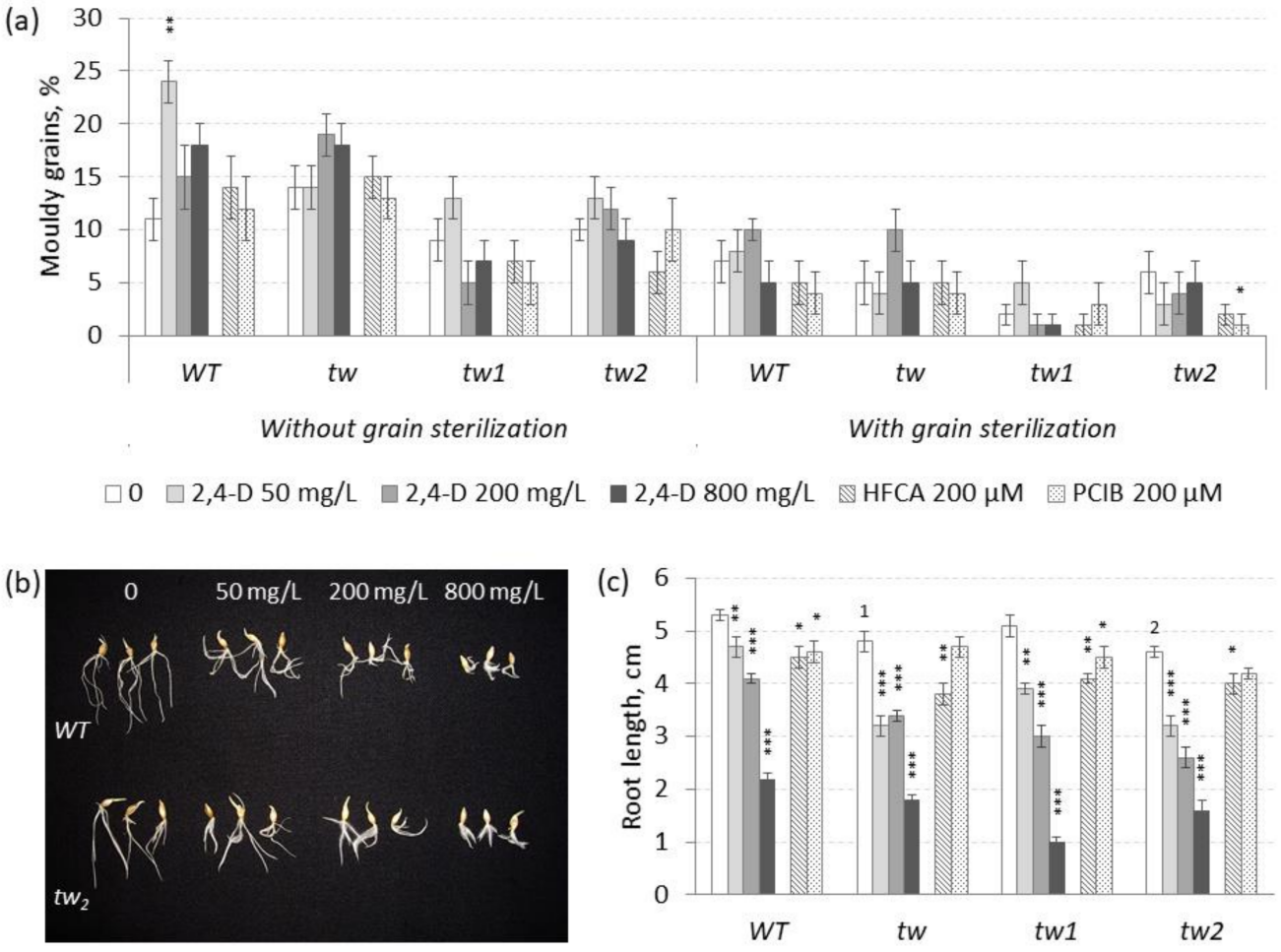

Figure 2. Effects of 2,4-D over a range of 50–800 mg L^{-1} and the auxin inhibitors HFCA and PCIB on (**a**) the frequency of mouldy grains (%) and (**b**,**c**) the root length (cm) of unsterilized germinating grains of *tw* mutants and the *WT*, as measured after 5 days. For mouldy grains, n = 10 *Petri* dishes (100 grains); for root length, n = 30. Grain was produced in 2013, and the MGG assay was performed in 2017. The asterisks represent significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) between the control and the 2,4-D treatment. The numbers denote significant differences (^{1} $p < 0.05$; ^{2} $p < 0.01$) between the controls of the *tw*-type mutant and the *WT*.

In the range of 10–50 mg L^{-1}, an appreciable inhibitory effect of 2,4-D on the root growth of allelic *tw*-type mutants depended on (1) the plant genotype and (2) the sterilization status of grains (Figure 1b and Table S1b). In most cases, 2,4-D significantly inhibited root growth only in unsterilized germinating grains of all tested genotypes, including the nonallelic *twN18* and *twmk* mutants. The *twmk* mutant was the most sensitive to 2,4-D among all tested *tweaky*-type mutants. In contrast, 2,4-D-induced root growth inhibition in sterilized germinating grains mostly in a nonsignificant manner (Figure 1b and Table S1b).

The tested lower 2,4-D concentrations (10–50 mg L^{-1}) revealed an interesting dependence of 2,4-D-induced root growth inhibition on grain sterilization status; consequently, the effect of elevated 2,4-D concentrations was investigated in further experiments. In the range of 50–800 mg L^{-1} 2,4-D, a significant effect of 2,4-D on MGG was observed only in unsterilized grains of *WT* ($p = 0.0026$), in which 2,4-D increased the level of MGG (Figure 2a and Table S2a), while in the range of 10–50 mg L^{-1} 2,4-D, a significant increase in MGG was observed only in sterilized grains of the tw_2 genotype ($p = 0.024$; Figure 1a and Table S1a).

In contrast to the effect of the lower concentrations, the higher concentrations (50–800 mg L^{-1}) of 2,4-D induced a significant decrease in root length independent of the plant genotype and the grain sterilization conditions, and the inhibitory effect of 2,4-D

on root growth was dose-dependent (Figure 2b,c and Table S2b). The root growth of the *tw* and tw_2 allelic mutants, but not the tw_1 allelic mutant, was weaker than that of *WT* germinating grains. An unsterilized grain background better revealed the inhibitory effect of 2,4-D on the germination rate, which was uniform independent of the plant genotype. In turn, grain sterilization revealed better differences among allelic mutants in the response to 2,4-D according to the germination rate (Table S2c). In general, 2,4-D in the range of 50–800 mg L^{-1} inhibited root length independent of the plant genotype (Figure 2c), while in the range of 10–50 mg L^{-1} 2,4-D, differences between *WT* and *tw*-type mutants and among allelic *tw* mutants themselves were observed (Figure 1).

Despite the proposed opposite effects to the action of 2,4-D, the auxin inhibitors HFCA and PCIB did not show a significant effect on MGG, except PCIB in sterilized grains of the tw_2 mutant, in which the MGG level decreased (Figure 2a and Table S2a). However, both auxin inhibitors suppressed root growth similarly to 2,4-D (Figure 2c and Table S2b).

Theoretically, the effects of TCA and SA are supposed to be opposite to those of 2,4-D, and TCA decreased the frequency of MGG in unsterilized germinating grains of the *WT* and all allelic *tw*-type mutants (Figure 3 and Table S3).

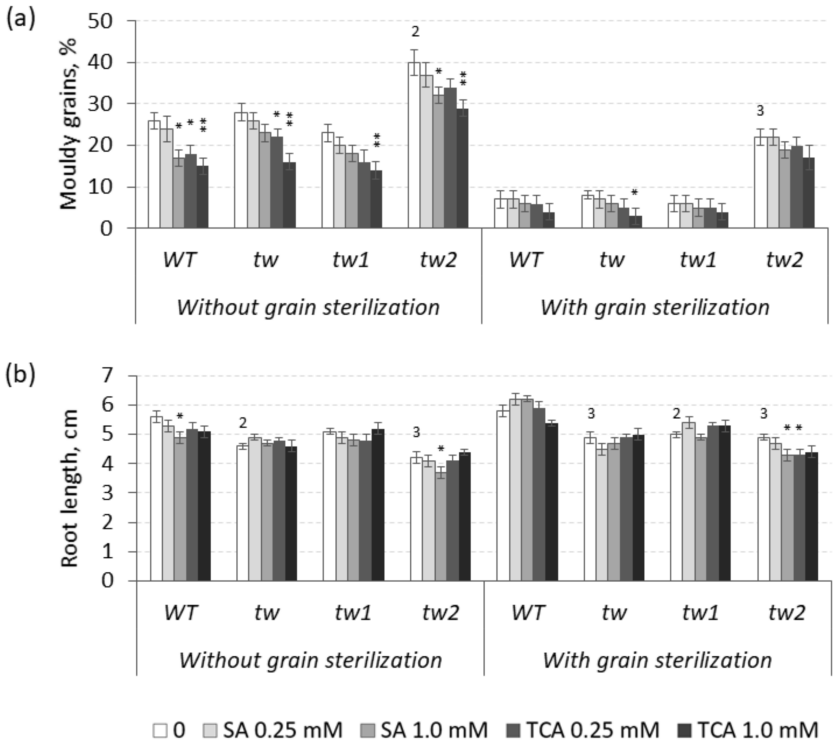

Figure 3. Effects of salicylic (SA) and *trans*-cinnamic (TCA) acids on (**a**) the frequency of mouldy grains (%) and (**b**) the root lengths (cm) of unsterilized and sterilized germinating grains of barley *tw* mutants and the *WT*, as measured after 5 days. For mouldy grains, n = 10 (100 grains); for root length, n = 30. Grain was produced in 2016, and the MGG test was performed in 2020. The asterisks represent significant differences (* $p < 0.05$; ** $p < 0.01$) between the control and the SA or TCA treatment. The numbers denote significant differences (^{2} $p < 0.01$; ^{3} $p < 0.001$) between the controls of the *tw*-type mutant and the *WT*.

However, in sterilized germinating grains, TCA decreased MGG at a significant level only in the allelic *tw* mutant, while SA decreased MGG at a significant level only in unsterilized grains of the tw_2 mutant (Figure 3 and Table S3a). Similar to 2,4-D, SA and TCA also inhibited root growth, but only in the allelic mutant tw_2 (Figure 3b and Table S3b). Neither compound showed any effect on the grain germination rate (Table S3c).

3.2. Fungi Spectrum in the Internal Grain Tissues of Barley *tw*-Type Mutants and Revertants

To reveal the possible differences in the fungal diversity in surface-sterilized mouldy germinating grains of tested barley genotypes, the spectrum of fungi species was investigated. Among the fungi that frequently reside in the internal tissues of barley grains, *B. sorokiniana* prevailed in all the tested plant genotypes (Figure 4 and Table S4). In addition to the *WT*, tw_1 and tw_2 mutants, the fungi spectra were also studied in several revertants that arose during the phase of stabilization of tw_1 and tw_2 mutants. The revertant studies allowed for a broader understanding of the differences between *tw*-type mutants and the *WT*.

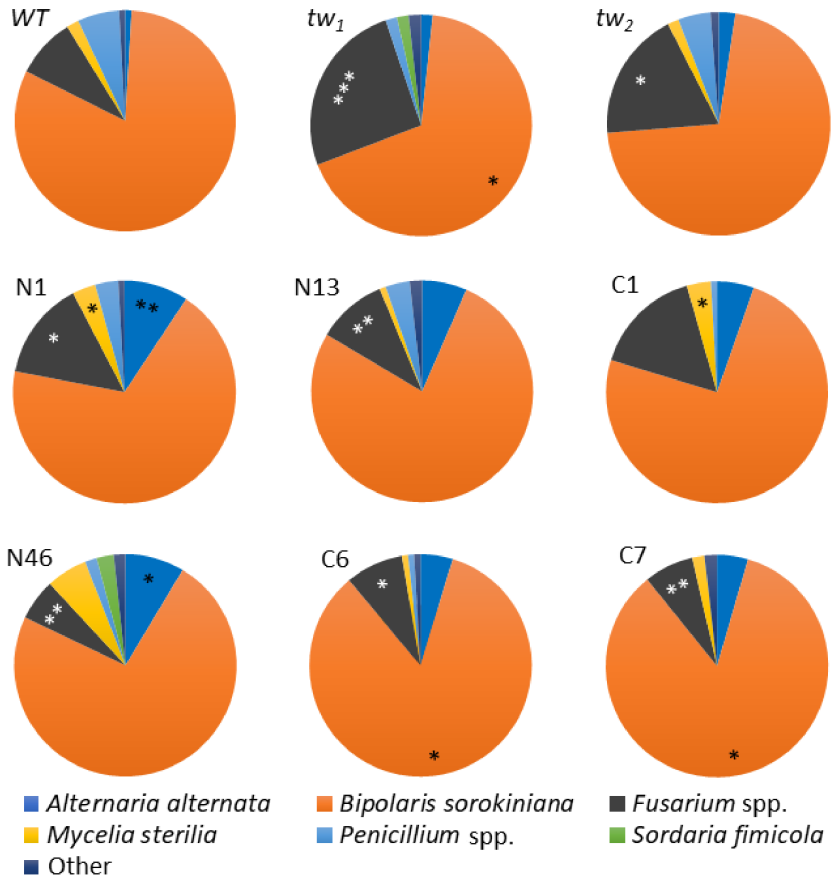

Figure 4. Spectra of fungi (%) in the internal grain tissues of barley *tw* mutants and the *WT*. The middle row—revertants from tw_1; the lower row—revertants from tw_2. N—revertants with normal spike and floral structure, C—revertants with normal floral structure but compactoid spikes. Grain was produced in 2013, and the analysis was performed in 2014. The asterisks represent significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) between the *tw*-type mutant and the *WT* (in the upper row) or between the revertant and the respective initial *tw* mutant (N1, N13 and C1 derived from tw_1, N46, C6 and C7—from tw_2).

In the grains of the tw_1 and tw_2 mutants, the *B. sorokiniana* frequency was lower than that in the *WT* strain, but the difference was only statistically significant in tw_1. Interestingly, the level of *B. sorokiniana* was only significantly lower in grains of revertant N1 compared to *WT* ($p < 0.05$) and remained the same as in grains of its parental mutant tw_1. Such a tendency was not observed. A similar tendency was also observed in other revertants, except for the compactoid (C)-type revertants from tw_2, but only in an insignificant manner. However, a decrease in the *Bipolaris* proportion occurred at the expense of the increasing *Fusarium* portion in the fungi spectra, and the observed effect was statistically significant (Figure 4). Comparable results were also obtained after analysing the fungi spectra in

the internal grain tissues in our previous studies [22]. This finding provided a pretext for studying the effects of grain meals made from the different *tw*-type allelic mutants and the *WT* on *B. sorokiniana* growth.

3.3. The Impact of Meals from Grains of tw-Type Mutants on the Colony Growth of Bipolaris sorokiniana

The growth of *B. sorokiniana* colonies on MEA media supplemented with meals prepared from the grounded dry grains of allelic mutants *tw*, tw_1, and tw_2 was compared with *B. sorokiniana* growth on MEA medium containing meals from the grains of *WT*. Additionally, the effects of the SA and TCA concentrations were investigated on such media (Figure 5). The meals from the allelic *tw* mutants significantly decreased the growth of *B. sorokiniana* colonies. In most cases, a further statistically significant decrease in *Bipolaris* colony growth occurred only after TCA but not SA addition. SA decreased the growth of *B. sorokiniana* in a concentration-dependent manner only in tw_2. TCA also showed a strong inhibitory effect on *B. sorokiniana* growth on MEA media with meals from the *WT*. Even the lowest concentration of TCA, 0.05 mM, significantly decreased the growth of *Bipolaris* colonies, and a further increase in TCA concentration did not enhance its inhibitory effect on *B. sorokiniana* growth in *WT* media (Figure 5).

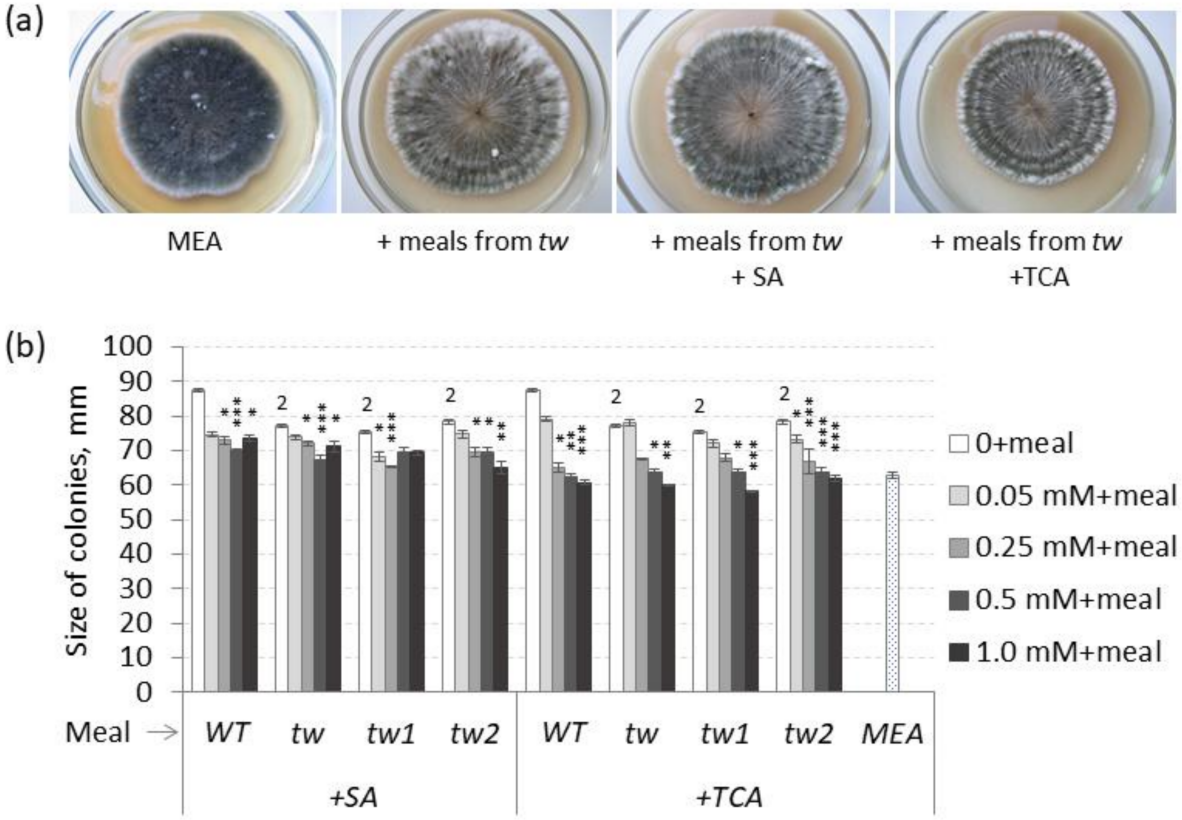

Figure 5. Effects of salicylic acid (SA) and *trans*-cinnamic acid (TCA) on the growth of *Bipolaris sorokiniana* after 7 days of growth on medium containing meal from *tw*. (**a**) The morphology of *B. sorokiniana* colonies grown on MEA media with different supplements. (**b**) Size of the colonies 7 days after inoculation. MEA, malt extract medium. Grain was produced in 2013, and the experiment was performed in 2014. The asterisks represent significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) between the control and the SA or TCA treatment. The numbers denote significant differences (^{2} $p < 0.01$) between the controls of the *tw*-type mutant and the *WT*.

4. Discussion

Multi-year studies of *tw*-type mutants have revealed the significant variation in MGG expression observed in the seed material from different years and statistically significant

differences in MGG levels between the allelic *tw*-type mutants (Table 1). Naturally, among the most important factors that influence the occurrence of fungal diseases is weather conditions during the growing season [27–29]. However, another source of such variations may lie in the highly variable expressivity of the *tw* phenotype. One of the main characteristics of *tw*-type mutants is the irregular transformations of lodicules, grass-specific floral organs, to sterile reproductive organs, which partially open the flowers and physically facilitate the penetration of pathogen spores and water into the developing grain [3]. Depending on the years, the fraction of aberrant flowers of mutants *tw*, tw_1, and tw_2 fluctuates in the ranges of 63–80%, 29–48% and 32–90% (respectively) [unpublished data], and such expressivity variations in flower structure from year to year can directly influence the quality of grains from different years.

Table 1. Variation in MGG frequencies (%) in barley *tweaky spike* (*tw*) allelic mutants in different years of seed material growth.

Year	Position in Spike	*WT*	*tw*	tw_1	tw_2	References
1992	Total u.s.	25 ± 4	42 ± 2 [3]	40 ± 5 [1]	38 ± 5 [1]	[4]
1993	Total u.s.	19 ± 4	33 ± 2 [2c]	21 ± 4 [c]	45 ± 5 [3c]	
1996	Total u.s.	11.0 ± 2.2	-	34.5 ± 3.4 [3]	28.5 ± 3.2 [3]	[19]
2002	Total u.s.	11.6 ± 2.3	18.0 ± 2.7	18.4 ± 2.8	19.2 ± 2.8 [1]	[21]
1999	Total u.s.	7.8 ± 1.0	18.7 ± 1.6 [3]	20.2 ± 2.9 [2]	17.0 ± 1.5 [3]	[30]
2012	Total u.s.	34.5 ± 2.9	-	32.0 ± 2.6 [c]	48.0 ± 2.5 [3c]	Unpublished data
2013	Total u.s.	11 ± 2	14 ± 2	9 ± 2	10 ± 1	Present study
2013	Total st.	7 ± 2	5 ± 2	2 ± 1	6 ± 2	[30]
2013	Total u.s.	15 ± 2	16 ± 2	8 ± 2 [1a]	20 ± 2 [b]	Unpublished data
2013	Total st.	4 ± 2	5 ± 2	1 ± 1	3 ± 2	Present study
2016	Total u.s.	24 ± 4	-	-	35 ± 2 [2]	
2016	Total st.	6 ± 2	-	-	20 ± 3 [2]	
2017	Total u.s.	6 ± 2	13 ± 2 [1a]	-	19 ± 2 [3a]	
2018	Total u.s.	8 ± 2	-	17 ± 2 [2]	22 ± 2 [3]	

[1,2,3] Comparison with the *Wild Type*: [1] $p < 0.05$; [2] $p < 0.01$; [3] $p < 0.001$. [a,b,c] Comparison of allelic *tw* mutants: [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$. u.s., unsterilized grains; st., sterilized grains.

The present study revealed a smaller than expected effect of the exogenous synthetic auxin 2,4-D and both auxin inhibitors HFCA and PCIB on MGG frequency in *tw*-type mutants, considering their dramatic rescue effects on variations in spike and floral structure (respectively), which were revealed in our previous works [8,9]. Such a negligible effect of 2,4-D and both auxin inhibitors on MGG frequency of different *tweaky*-type mutants, strongly suggests that auxin pathway disturbances in *tw*-type mutants are generally restricted to the spike and floral structure but not to germination events.

A notable feature of the grain material used in the present study is the pesticide-free production mode, which was applied for many years. Under the background of high natural infection, the most effective means against the moulding of germinating grains is widely used grain surface sterilization [31–33], which was effective independently of the plant genotype and seed material from different years. However, a comparison of sterilized and unsterilized seed material appeared to be an effective strategy for the auxin–pathogen relationship study. A significant increase in the MGG level was only determined after 2,4-D treatment of unsterilized *WT* grains (Figure 1a, Table S1a), while the inhibitory effects of salicylic acid and especially *trans*-cinnamic acid on the MGG frequency were also statistically significant only for unsterilized germinating grains of the tested plant genotypes (Figure 3a). Comparison of sterilized and unsterilized grains in the MGG assay

also revealed differences among the allelic mutants *tw*, tw_1, and tw_2 in response to 2,4-D. Allelic differences in the pathogen response related to auxin action are poorly studied. Stotz et al. [34] determined differences among allelic mutants in the transcriptional repressor *auxin response factor 2* (*arf2*) gene in response to the ascomycete *Sclerotinia sclerotiorum*. From the multi-year studies of *tw*-type mutants, it was concluded that tw_2 is the most sensitive to MGG (Table 1). Comparison of sterilized and unsterilized grains in the present analysis also confirmed tw_2 to be the most responsive to 2,4-D in the MGG assay (Figures 1 and 2).

The increase in MGG frequency after 2,4-D treatment in some cases of the present study (Figures 1a and 2a) coincides with the known auxin effects on phytopathogens. Endogenous auxin was demonstrated to enhance the invasion of biotrophs and to inhibit the development of necrotrophs [13,35–37]. In contrast, exogenously applied auxin, in general, showed the opposite effect when the invasion of nectrotrophs was escalated after auxin application [38,39]. The predominant internal fungi in all tested barley genotypes belonged to the *Bipolaris* and *Fusarium* genera (Figure 4), both of which are considered hemibiotrophic, exhibiting a brief biotrophic phase before switching to the dominant necrotrophic phase [40,41]. This conclusion is also supported by the inhibitory effects of SA and TCA on MGG revealed in the present study. It was demonstrated that SA suppresses pathogen growth in plants through the repression of auxin signalling [42,43]; therefore, the SA and auxin pathways act in a mutually antagonistic manner during plant defence responses [13,35,44,45]. TCA, one of the precursors of SA, also has antifungal properties and is considered a natural protective substance against phytopathogens [46,47]. TCA was long considered an auxin transport inhibitor, but recent works have suggested that another isomer, *cis*-cinnamic acid, but not TCA, is a potent inhibitor of auxin efflux, hence the exact physiological activity of TCA has remained a matter of debate [48].

The effects of *tweaky spike* mutations on fungi spectra are also notable. In internal grain tissues of all tested barley genotypes, the prevailing fungus in the spectra was *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*) (Figure 4). This agrees with recent publications on fungal spectra in barley grains in the Baltic region [49,50]. *B. sorokiniana* causes severe diseases and significant yield losses mainly in warm humid growing regions; nevertheless, it has spread towards the Northern Hemisphere [6,51]. The present study shows that its spread strongly depends on the plant genotype. In both *tw*-type mutants and most revertants tested, a decrease in the *B. sorokiniana* proportion and an increase in the *Fusarium* spp. proportion occurred in the fungi spectra (Figure 4). This trend suggests that the grains of *tw*-type mutants contain substances that are unfavourable for *B. sorokiniana* growth. The following supposition was also confirmed by *B. sorokiniana* growing on media supplemented with meals from individual *tw* allelic mutants and the *WT*. A decrease in *B. sorokiniana* in the fungal spectra of *tw*-type mutants coincides with the growth inhibition of *B. sorokiniana* colonies on media with the addition of meals from all allelic *tw* mutants (Figure 2).

The present study showed that more appreciable conclusions on the action of the modifying factors could be made when the MGG assay is performed in parallel with the root growth test. The inhibition of root elongation of germinating grains is one of the earliest and most distinct symptoms exhibited in response to auxins, especially 2,4-D [18], but auxin promotes lateral root formation [13], and this auxin feature is related to pathogen invasion [17]. In the present study, significant differences in 2,4-D-induced root growth inhibition among the different allelic *tw*-type mutants were revealed only in the lower range (10–50 mg L^{-1}) of 2,4-D concentrations (Figure 1b). Moreover, significant root length inhibition with 2,4-D in the allelic mutants *tw* and tw_2 and the nonallelic mutant *twN18* was only observed in unsterilized germinating grains, whereas in sterilized grains, root growth inhibition in these mutants was absent (Figure 1). While differences in MGG frequency between sterilized and unsterilized grains are naturally expected, the dependence of root growth after 2,4-D treatment on grain sterilization status was quite an unexpected phenomenon. Hypochlorite-based agents, including commercial bleach, are routinely used for surface sterilization of various plant materials [32,52]. Hypochlorite was

proposed to react with the seed surface, forming a chlorine cover that is not completely removed by rinsing, and subsequently can be converted into highly toxic chloramines that easily penetrate plant tissues [32]. Furthermore, various salts are known to antagonize the phytotoxicity of several herbicides, including 2,4-D [53,54]. After surface sterilization with commercial bleach, nonremovable chlorine compounds can antagonise exogenously applied 2,4-D and subsequently diminish the effect of root growth inhibition in comparison to that of unsterilized 2,4-D-treated grains. This observation highlights the importance of comparing sterilized and unsterilized grain conditions in studies of plant-auxin–pathogen relationships where the action of exogenous auxin will be analysed in germinating grains since hypochlorite-based sterilization itself can lead to underestimation of the 2,4-D effect on root growth.

5. Conclusions

Our findings allow us to reach a number of conclusions. The effect of exogenous 2,4-D on barley MGG coincides with the role of auxin in the invasion of necrotrophs, but its effect on MGG was found to be lower, as may be expected based on the effects of 2,4-D on spike structure.

The effects of SA, TCA, and 2,4-D on MGG depended on the grain sterilization status.

The *tweaky spike* mutation caused variations in the fungi spectra in the germinating barley grains by decreasing the *B. sorokiniana* and increasing the *Fusarium* spp. proportions. This result agrees with the negative effects of meals from the tw_2 mutant on *B. sorokiniana* growth.

The effect of low doses of 2,4-D on barley root growth depended on the grain sterilization status, suggesting hypochlorite-based surface sterilization methods should be used with caution in studies where the action of exogenous 2,4-D will be analysed in germinating grains.

A group of *tw*-type barley mutants is a convenient model for the investigation of immunity-related substances in barley MGG.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11112180/s1, Table S1. Effects of 2,4-D over a 10–50 mg L^{-1} range on the frequency of mouldy germinating grains (a), root length (b) and grain germination rate (c) of barley *tw* mutants and the *WT*. (d) *p* values of differences between sterilized and unsterilized grains under different experimental conditions. Table S2. Effects of 2,4-D over the 50–800 mg L^{-1} range and the auxin inhibitors HFCA and PCIB on the frequency of mouldy germinating barley grains (a) root length (b) and grain germination rate (c) of *tw* mutants and the *WT*. (d) *p* values of differences between sterilized and unsterilized grains under different experimental conditions. Table S3. Effects of salicylic acid (SA) and *trans*-cinnamic acid (TCA) on the frequencies of mouldy germinating grains (a) root length (b) and germination rate (c) of barley *tw* mutants and the *WT*. (d) *p* values of differences between sterilized and unsterilized grains under different experimental conditions. Table S4. Frequency of fungi in the internal grain tissues of barley *tw* mutants, revertants and the *WT*. Figure S1. Spikes of barley genetic material used in the present study. From left: *WT* (cv. Auksiniai II), *tweaky spike*, *tweaky N18*, *tweaky and missing kernel*; and revertants: C, compactoid and N, normal spike shape.

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