Appendiks 4:

Water based heat treatments to control *Tilletia tritici* in wheat in organic agriculture

Manuscript submitted to Plant Disease July 2000

by

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Abstract

Common bunt (*Tilletia tritici*) is a serious disease in organic wheat production, due to its strong potential for rapid proliferation. This study investigated the potential of the hot-water method (dry seeds in hot water without presoaking) as a method for controlling common bunt in organic agriculture, by emphasizing the control effect as well as the side-effects on seed germination. Effects of washing and of time-temperature combinations were investigated. Four years of field experiments and one greenhouse experiment were carried out from 1992 to 1997. Hot-water temperatures ranged from 50 to 65°C and the treatment duration from 0.5 to 7 minutes. Results show that, by hot-water treatment, disease can be reduced by more that 95% at temperature-time combinations from 50°C-3 minutes (minimum) to 65°C- 3 minutes, without significant reduction in germination performance. Thus, hot-water treatment without presoaking provides an option for controlling common bunt in organic wheat production.

Additional keywords: ecological agriculture, germination speed, mean germination time, organic farming, seed treatment, Tilletia caries, thermo-therapy, warm water

Introduction
History of thermotherapy. Common bunt is caused by the pathogen *Tilletia tritici* (Berk.) Win syn. *Tilletia caries* (D.C.) Tul. Allready Olivier de Serres in 1600 recommended dipping seeds in a water bath to remove bunt balls and spores (7), and in 1755 Tillet proved that bunt was caused by "the black dust" even though he did not believe that it was a fungus (26).The water-based thermotherapy treatment as a phytosanitary method was invented by the Danish agronomist J.L. Jensen in the 1870-80s, originally for controlling late blight (*Phytopthera infestans*) in potatoes by treating the tubers. In 1888 Jensen published his first paper on hot-water treatment of common bunt in wheat (12). From about 1890's to about 1930, several papers were published on the effect of different seed treatment methods, including the hot-water method, recommending the method for a wide range of pathogens, including common bunt (*Tilletia tritici*) of wheat (see e.g. 17, 26). Among those papers, Lind (17) provided one of the most comprehensive studies on the effects of the hot water method. From the late 1920's water-based thermotherapy was replaced exclusively by chemical seed treatments, except in the case of loose smut (*Ustilago nuda*) in barley and oats, where the warm-water method was used by seed companies until the late sixties (13).

Organic agriculture. Organic agriculture is developing significantly in Europe these years (15). From the research point of view an increased focus on different problems related to cultivation practices in organic agriculture has been the result. One of these problems is common bunt (*Tilletia tritici*) in wheat. Under Danish conditions the incidence and potential severity of this pathogen has been discussed in several reports (6,10). A survey made by Winter *et al.* (24) on seed borne diseases in organic farming and found problems maintaining sufficient seed quality, including problems with common bunt. In Germany, a Ph.D.-thesis by Piorr (19) thoroughly covered the subject of seedborne diseases, including common bunt, in winter wheat grown under organic conditions. The study of Piorr (19) focused on spread of the disease, resistance and possible control measures suitable for organic agriculture, including hot- and warm-water treatment. All studies indicate that common bunt is potentially the worst seedborne disease in organic wheat growing. At present there is an urgent need in organic agriculture for the development of appropriate alternatives to current chemical seed treatments.

Hot- and warmwater-method: The principles of thermotherapy are excellently described by Baker (1), whose paper covers thermotherapy of both plants and seeds. Piorr (19) divided water based thermotherapy into warm-water ("Warmwasser") and hot-water ("Heisswasser") treatments. Warm-water treatments is conducted at temperatures below 50°C and for periods of 0.5-4 hours. Hot-water treatments is conduced at higher temperatures (50-65°C) for shorter periods of 2.5 to 20 minutes, but include pre-soaking at room temperature for 2.5 hours. To our knowledge no fixed definition of warm- or hot-water treatment is available or agreed, but we suggest that hot-water is used only for treatments without presoaking. Piorr (19) studied the effect of hot- and warm-water treatment on the germination of spores of *Tilletia tritici* and the germination of wheat seeds, and found that warm-water treatment at 50°C for 60-90 minutes reduced spore germination to below 10% of the control, but the germination percentage of seeds was also reduced by 10-20%. Hot water treatment at 55°C for 7.5 -10 minutes (with pre-soaking for 2.5 hours) also reduced spore germination to below 10% of control. Unfortunately seed germination percentage was also decreased to about 10% of the control. Winter *et al.* (24, 25) studied the effect of warm-water treatment (2 h, 45°C) on the attack of common bunt in three years of field trials, and found a reduction between in average 71-80%, without any negative effects on field emergence.

It is not obvious why pre-soaking is needed to control common bunt, since the bunt spores are loosely attached to the seed surface, and consequently not embedded in any seed structures as happen in *Ustilago nuda*. The risk of seed damage from heat treatment increases with increasing moisture content in the seeds (1, 8). Our own results (unpublished) show minimal reduction in germination speed in samples with a water content of 10% (wb) before hot-water treatment at 55°C for 5 minutes, whereas there were significant reduction with increasing water content. Thus, an attempt to reduce or keep seed water content low would reduce side-effects and benefit heat treatments.

Beside the studies in the beginning of this century, several more recent studies on different alternative control measures have been made (2, 4, 5, 19). During the last few years hot water treatment has been re-considered as a environmentally friendly method to control seed borne pathogens. Germination of teliospores of *Tilletia indica* after hot-water treatment gave a reduction compared to water treatment at 25°C of approximately 88% and 95% following 1 and 5 minutes at 60°C, respectively (21). Teliospore (*T.indica*) and wheat seed germination were tested by Smilanick *et al.* (20) at 54°C for varying periods between 0 and 20 minutes, who found approximately a 65% reduction in spore germination, and no reduction in seed germination after 10 minutes treatment. Other methods like electron beams have been tested for prevention of germination of teliospores of *T.tritici* and *T.contraversa*, but treatments causing reduction in spore germination also caused servere reduction in seed germination (22). Several papers concerned with hot water treatment in relation to other different host/pathogen relations has been published during the past few years, for example Maize/*Fusarium moniliforme* (9), Wheat and Medicago/ *Fusarium avenaceum, F. graminearum* (16).

In order to study the potential of the water based thermotheraphy against *Tilletia tritici*, not only from the point of view of maximally controlling the pathogen, but also from the point of view of minimal harmful effects on the seed germination properties, we concentrated on hot-water treatments (without presoaking) only. From this starting point, the objective of this study is toinvestigate possible improvements of the hot-water method, providing the basis for an effective method, suitable for control of common bunt in organic agriculture.

Materials and methods

In the period 1992-1997 one climate-chamber/greenhouse experiment and four field experiments with hot-water treatment against *T.tritici* were made at the Royal Veterinary and Agricultural University Research Farm, 20 kilometres west of Copenhagen, Denmark. The soil type is a moreanic sandy loam.

First trial (field 1992-1993)

Treatments and design: The aim of this trial was to test different treatments of alternative control methods, including hot-water, milk-, mustard- and wheat-flour applications. The field experiment was designed as a balanced complete randomized experiment with 6 m^2 plots in 4 replications. Only the part concerning the hot-water method is presented in this study. Hot-water treatments were made in a plastic container, with the temperature kept constant by adding small amounts of boiling water. Winter wheat variety Kosack naturally contaminated with a high level of spores was used, and approximately 1 kg was poured into 10 litres of hot water at 55°C with manually stirring throughout the treatment period. After treatment samples were cooled in cold tap water for approximately 2 min. All treatments was made in a room separated from rooms which contained contaminated seeds. Howerver, when the experiments where sown the same sowing machine was used, allowing some re-contamination from other treatments/control treatments with higher level of contamination. Comparison of the results from first and second field experiment, where another procedure was used (see below and Table 1) show, that this procedure was not a problem. At the start of July (Zadoks growth stage 73-77) results were assessed by counting the number of infected and non-infected ears. Partially infected ears were recorded as infected. All together five to ten 4-m rows in each plot was counted, corresponding to 1500-2000 ears per replicate.

Statistical methods: The statistical analysis of this kind of data must be able to handle the non-normally distributed data, as well as the inhomogene variance arising from some treatments with very low levels of attack and some treatments higher levels of attack. Further, concerning *T.Tritici* the treatment efficiacy, i.e. reduction compared to control, is the major parameter of importance. Consequently, results were analyzed by the procedure PROC GENMOD in SAS version 6.12 (SAS Institute, USA), using a logistic link-function and a binomial distribution. This takes into consideration that the logit transformation (%disease/(100-%disease)) homogenizes the variance and pays attention to the fact that disease data belong to a binomial distribution. Data were analyzed in terms of number of diseased ears relative to number of ears examinated (percentage disease). Results are presented as percentage disease ±

95% confidence limits. Percentage disease is estimated by back-transformation of logit-values from the model-output. Confidence limits are calculated from the covariance matrix, by calculating the variance (s.e.), multiplied by the corresponding t-value from the t-distribution. Calculation of upper and lower confidence limits were made on logit-transformed values. Subsequently upper and lower logit-estimates of confidence limits were back-transformed as (estimate=$100 \times e^x/1+e^x$), where x is logit-estimate from the model. For overview and treatment comparisons results are presented as the percentage reduction compared to control. The control value in this case is not a fixed value, since the level of diseased ears in untreated plots varies, calculation of confidence limits on reduction percentages takes the variation in the control into consideration. Confidence limits on the reduction percentages was calculated as (14):

$$confidence\ limit_{95} = z\sqrt{CV_x^2 + CV_{control}^2}$$

z = $\text{estimate}_{x\ (\%\ disease)}/\text{estimate}_{control\ (\%disease)}$
CV_x = $100 \times \text{deviation}_x/\text{estimate}_x$
$CV_{control}$ = $100 \times \text{deviation}_{control}/\text{estimate}_{control}$

This method can result in reduction percentages above 100% (disease reduction) and below 0% (germination and germination speed reduction). Results of the first experiment are presented in Table 1.

Second experiment (field 1993-1994)

Treatments and design: The aim of this experiment was to repeat the first experiment in order to examine the stability of the method, and to compare the hot-water treatment to rinsing in clean and soapy water, respectively. Washing in water was done by letting tap water run in a 10 liter container, while stirring in the seeds until the water was visually “clean” (not muddy). Soap water treatment was done in several batches (of 10 liters) of soapy water, finishing with clean water. The field experiment was laid out as a balanced block trial with 6 m² plots in 4 replicates. Treatments were made in the same way as in the first experiment. In the field, plots with hot-water treated seeds was placed systematically to avoid undesirable contamination. No lab-germination tests were made, but field emergence was counted 3 weeks after sowing.The sowing date was October 1, 1993.

Statistical methods: Results were recorded, analyzed and presented as described for the first experiment. Field emergence was analyzed by PROC GLM in SAS version 6.12 (SAS Institute, USA), using a one-way analysis of variance and the Ryan-Einot-Gabriel-Welsch multiple-range test to examine comparisons. Results are shown in Table 1.

Third experiment (Climate chamber 1995)

Treatments and design: Infected seeds (variety Kosack) were treated at six different temperatures (20, 40, 50, 55, 60 and 65°C) and compared to a control. 50 seeds were sown at 3 cm's depth in pots in a standard soil mixture (Pindstrup whole mixture II). Pots were placed in a block design in a climate chamber in 6 replicates. Climate chamber settings were 12 hours day and 12 hours night. Day-temperature started at 10°C and was decreased by 1°C every week, night temperature was 5°C throughout the experiment. Lights (light intensity was 250-400 μEm^2s^{-1}) were turned on only after the first germinated seeds were visible, and number of germinated plants was counted every day for six days after the onset of germination. After 5 weeks pots were kept at 5°C constant temperature for 46 days to ensure vernalization. After vernalization, pots were transferred to a greenhouse at constant 20°C and natural daylight.

Statistical methods: Assessment of disease and statistical analysis were made in the same way as previously described. Mean Germination Time to achieve 25% (MGT_{25}) of total sown seeds was determined in a laboratory germination experiment. Daily counts of 4x100 seeds were made in a germination assay conducted as a Hiltner test (11), but slightly modified using a constant lower temperature regime of 10°C. Germination data was fitted to a logistic function by PROC NLIN in SAS version 6.12 (SAS Institute, USA), and subsequently a Mean Germination Time to 25% germination was calculated from the fitted curve. Goodnes of fit was evaluated by a plot of standardized residuals, which showed an even distribution of residuals at different predicted values, and by calculation of the adjusted r^2 for the individual observations. In all observations the r^2-value exceeded 0.993. Further analysis of the MGT_{25} was done by PROC GENMOD in SAS version 6.12 (SAS Institute, USA), using no transformation (option: link=id) and the normal distribution as the chosen distribution. Confidence limits were calculated in the same way as for disease and germination reduction. Results are presented in Figure 1.

Fourth and fifth experiment (1995-1997)

Treatments and design: After promising results in our preliminary studies, we decided to make more thorough investigations, regarding the possibilities for optimizing the treatment efficacy of the method, especially concerning the interaction between temperature and duration of treatment and side-effects on germination. Treatments were made individually in the following way. Fourth experiment: Samples of 150 grams naturally infected winter wheat (variety Kosack) was placed in a basket of perforated metal sheets, allowing easy flowing of water, but hindering the escape of the seeds. Treatments were made in a water bath containing 26 litres, with continuously circulation of water and regulation of the temperature (±1°C). The basket, hands etc. was cleaned after each treatment. In the fourth experiment five temperatures (0, 20, 50, 55 and 60°C) and five treatment times (0, 1, 3, 5 and 7 minutes) were arranged in an unbalanced factorial experiment (see below). The fifth experiment was basically identical, except that 0, 0.5, 1, 3, and 5 minutes treatment times were used, and a treatment temperature of 65°C was added.

In both experiments, treatments were carried out systematically starting with the highest temperature and the longest treatment time (60(65)°C, 7 min.). Hereafter, the next treatment time (5 min.) at the same temperature, next temperature and so on, in order to minimize the risk of re-contamination with viable bunt spores. The water bath was emptied and cleaned between each treatment temperature. All treatments at 20°C were the last to be made. Seed samples were cooled two minutes in tap water, and placed on a paper-towel for predrying for approximately 30 minutes. Hereafter, samples were placed in perforated plastic bags ("bread bags") and placed in a drying room with constant air flow (20°C) for final drying. In the fifth experiment, water content (wb) was determined immediately after treatment, and after final drying.

The field experiment was laid out as an unbalanced factorial experiment, with eight replicates (fourth experiment) and four replicates (fifth experiment) of single 8 meter long rows. The unbalanced design was due to the control treatment only appearing once instead of for all combinations of treatment time and temperature. To avoid re-contamination, each row (plot) was placed systematically, in the same way as treatments were made, starting with the highest temperature and the longest treatment time (60°C, 7 min.). Control plots were sown last. The experiment was sown with single row sowing machine (Stanhay Robin), with removable seed container. After sowing each treatment, the seed container was blown clean by a compressor, in order to minimize contamination. Results were assessed in the same way as in the first field experiment. The fourth experiment was sown 26-27/9 1995, and the fifth experiment was sown 14-15/10 1996.

Statistical methods: Results on disease, germination and MGT from the two years were analyzed in the same way as previously described. Non-significant interactions was removed from the model before calculation and back-transformation of estimates. Results are presented in Table 2, Figure 2a-e and Figure 3.

Results

First and second field experiment

Results of first field experiment showed that the "traditional" hot-water treatment of 55°C for 5 minutes was very effective, reducing infection more than 99% (Table 1). Second year hot-water treatment reduced bunt-attack by more than 96%, and washing alone reduced it by approximately 88%. Field emergence in control was 263 plants/m² corresponding to an emergence rate of 93%. No significant differences was observed in field emergence.

Table 1: Results of first and second field experiment. Within each year of experiment treatments with different letters are significantly different at logit-transformed data ($p \leq 0.05$) evaluated by the contrast-option in the PROC GENMOD procedure. Percentage attack and percentage reduction is based on back-transformed values and indicated by 95% confidence limits, see text for details.

Treatment	No. of ears	Attack (%)	95% conf. interval (%)	**Reduction** [1] (%)	95% conf. interval (%)	Plants/-m² [2]
1992-93						
Control	4713	66 *a*	64.0-68.7	**0**	0	-
Hot-water treatment	5158	0.5 *b*	0.3-1.0	**99.2**	98.8-100.0	-
1993-94						
Control	4759	45 *a*	41.5-48.6	**0**	0	263 *a*
Washing	4345	7.9 *b*	6.1-10.1	**82.6**	78.4-87.7	240 *a*
Washing in soap water	4387	5.4 *b*	5.3-5.6	**87.9**	86.9-88.9	258 *a*
Hot-water treatment	4331	1.6 *c*	0.9-2.9	**96.4**	94.8-99.2	292 *a*
Sibutol LS 280	4591	0.1 *d*	0.0-1.2	**99.9**	99.7-102.4	-

(1) Reduction is compared to control
(2) - = no results.

Third experiment

Altogether 1053 ears were examinated in this climate chamber/greenhouse study. The effect of temperature on disease was significant ($p=0.0001$), as was the effect of temperature on germination percentage ($p=0.0001$) and on MGT_{25} ($p=0.0001$). At 50°C and 55°C there were high reduction percentages, not significantly different from 100% reduction, and no side-effects on germination were observed. Results are presented in Figure 1.

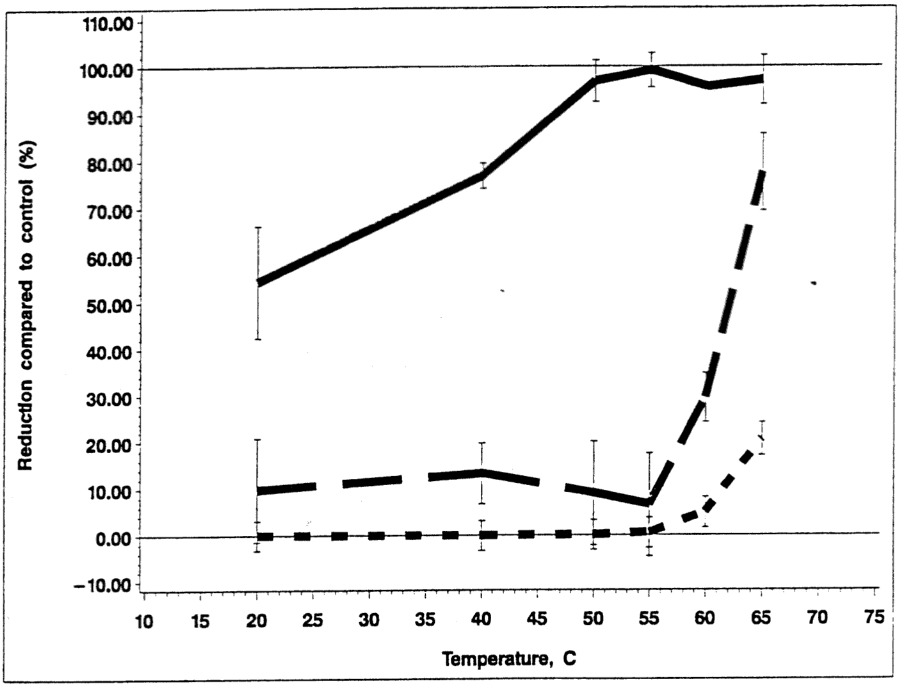


Figure 1: Results from third experiment. Reduction in the level of disease (——), reduction in germination percentage (- - -) and reduction in MGT_{25} (...) as compared to control. Bars indicate 95% confidence limits. Observations in control: 68.0 % diseased ears; germination percentage was 83.0%; MGT was 12.0 days.

Fourth and fifth experiment

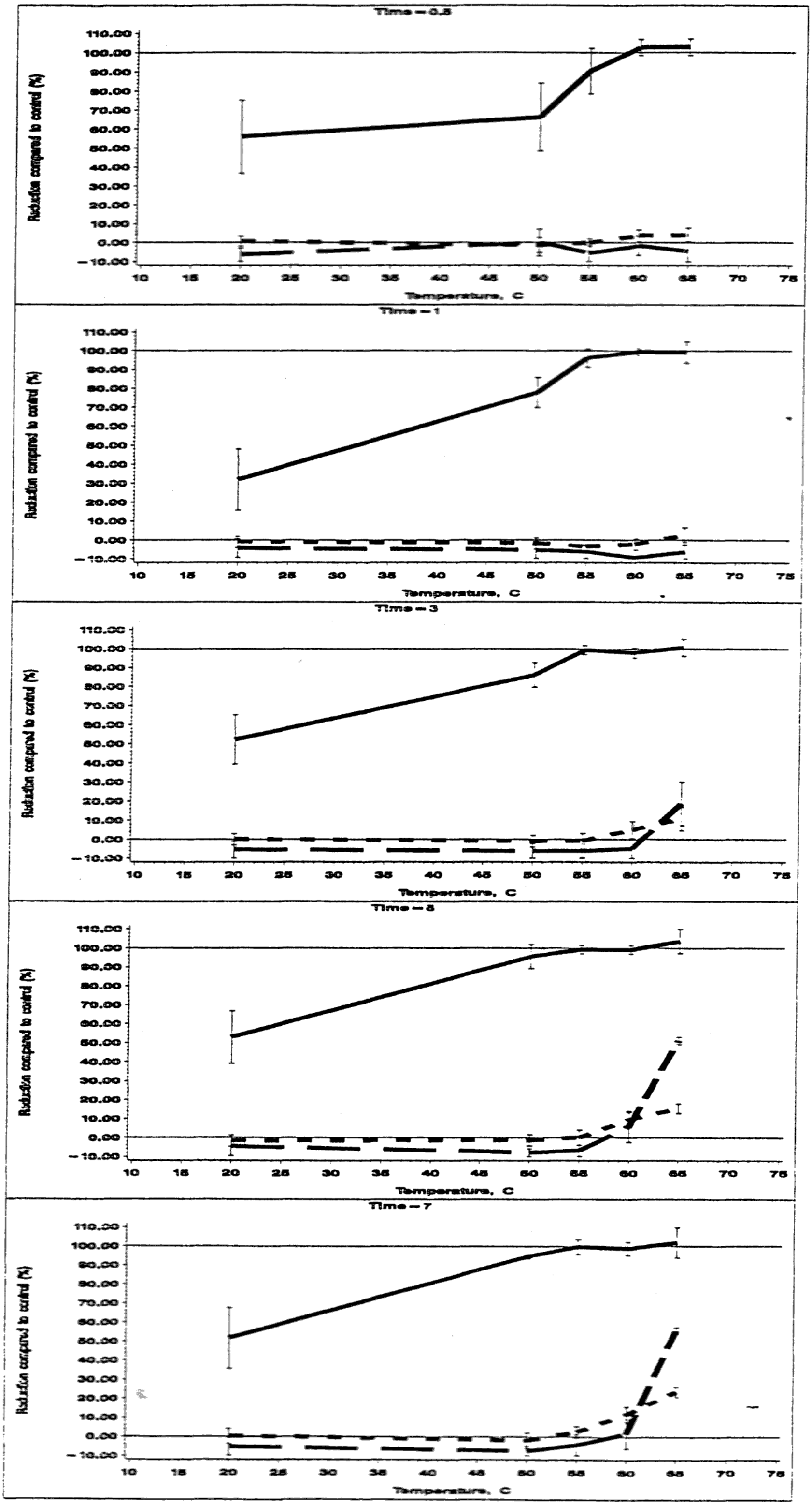
The number of ears counted ranged from 1045-1455 per treatment in the fourth experiment, and from 326 to 1448 in the fifth. All together 42,459 ears were included in the analysis. Statistical results of the analysis is presented in Table 2.

Concerning the level of disease both treatment temperature and treatment duration have significant impact on the results, since the temperaturextime interaction is significant (p=0.0066). Germination proporties are also significantly influenced by both treatment temperatrre and treatment duration (p=0.0001). Thus, our interpretation of the results are that both temperature and time are crucial parameters in an overall optimization of the hot-water treatment, aiming at maximal disease control and minimal side-effects on germination proporties. The level of disease in control treatment were significantly different in the two years. This is very common and can be ascribed to seasonal/climatic variation rather than to effects of infection level or other experimental factors. In both years an infection level higher than 5 grams of spores pr. kg seed was used, which under optimal conditions for infection could cause disease levels of about 80% infected ears. The year-effect was even more pronounced for germination proporties. The germination percentage was less and germination speed was slower in '96, and the side-effects on germination properties began at 60°C in '96, whereas it only began at 65°C in '97. These differences are ascribed to different periods from treatment to germination assay in the two years, and not to different effects of the treatment.

Table 2: Results of the type3 statistical analysis in PROC GENMOD of fourth and fifth experiments.

Effect		Disease	%germination.	MGT_{25}
	dF	Pr >F	Pr >F	Pr >F
year	1	0.0326	0.0001	0.0001
block(year)	6	0.0001	0.1481	0.0004
temperature	4	0.0001	0.0001	0.0001
time	4	0.0831	0.0001	0.0001
temperature×time	15	0.0066	0.0001	0.0001
temperature×year	3	0.1706	0.0017	0.0001
time×year	2	0.8121	0.1756	0.1984
temperature×time×year	6	0.2931	0.2444	0.0002
Average level in control		19.2	79.7	10.9
-1996		29.8	77.2	11.8
-1997		11.5	81.8	10.1

Figure 2:(A-E) Results from fourth and fifth experiment. Reduction in the level of disease (——), reduction in germination percentage (- - -) and reduction in MGT_{25} (…) as compared to control. A=0.5 minute; B=1 minute; C=3 minutes; D=5 minutes and E=7 minutes. Bars indicate 95% confidence limits. Observations in control can be seen from table 2.



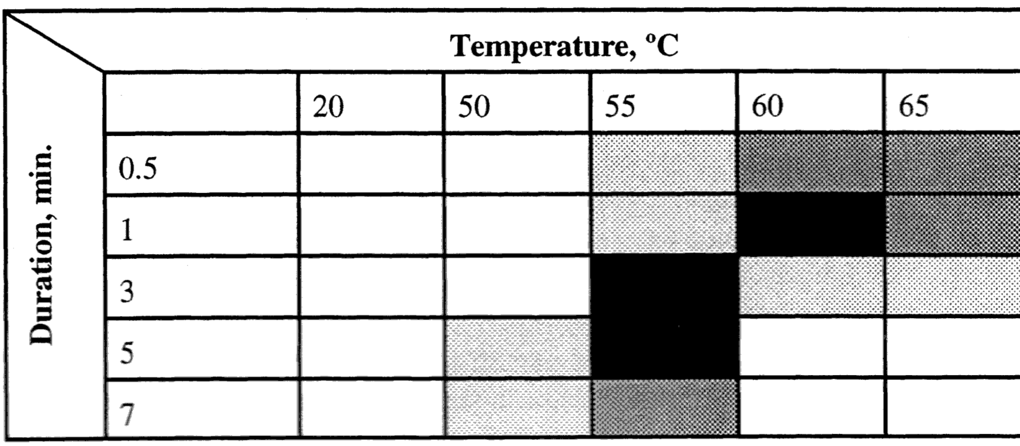
Thus, from these results there seems to be a window for safely hot-water treatment of seeds aiming at control of common bunt, and that a range of temperature-time combinations can be used to obtain satisfactory results.

Figure 3: Treatment window. Results from fourth and fifth experiment. Figurs illustrate minimal, average and maximal treatment range, in order to obtain satisfactory effects from three different sets of criteria:

Minimal: Lower limit>95% reduction in disease and upper limit<5% reduction in germination and upper limit<5% reduction in MGT (black).

Average: Mean>95% reduction in disease and mean<5% reduction in germination and mean<5% reduction in MGT (medium grey).

Maximal: Upper limit>95% reduction in disease and lower limit<5% reduction in germination and lower limit<5% reduction in MGT (light grey).



The criteria used in Figure 3 (>95% reduction and <5% reduction respectively) is chosen because variation in estimates in most cases would cause a 100% reduction to be part of the confidence interval when the true estimate is ≥95% reduction (when approx. 1000 ears per treatment are counted). Similarly 0% reduction would in most cases be included in the confidence interval, when the estimate of reduction is ≤5%.

In connection to the last field experiment, the water content of the seeds were measured just after treatment and just before sowing (Table 3). Within the range of relevant treatment temperatures (50-65°C), only treatment duration influenced water content of the seeds. Thus, from the point of view of re-drying, a shorter treatment duration is preferable, and time-temperature combinations which simultaneously optimize treatment efficacy, minimize negative side-effects on germination, and reduce re-drying costs should be chosen.

Table 3: Water content (% wb) in seeds after hot-water treatment (fifth experiment).

		Temperature, °C						average
		0	20	50	55	60	65	
Time (min)	0	15.9						15.9
	1		19.2	22.8	21.6	20.1	20.9	20.9±0.6
	1		21.3	21.8	23.1	21.2	23	22.4±0.3
	3		20.9	24.3	24.7	23.7	24.1	23.7±0.6
	5		21.1	24.6	24.6	24.8	25.5	24.4±0.6
average		15.9	20.7±0.5	23.4±0.7	23.8±0.5	22.4±1.1	23.7±0.7	

In this case, water content in the control was relatively high even in control (15.9%), and it can be expected that final water content just after treatment as well as germination damage would be reduced if the starting point was lower, such as e.g. 10-12% moisture content. After drying and before sowing there were no differences in moisture content between treatments (average 13.8%), except that the control level was 12.5%.

Discussion

The overall finding of these investigations is that the hot-water method provides an efficient pesticide-free tool for controlling common bunt in wheat in organic farming. Treatment efficacy is comparable to current chemical methods, with reductions above 95%, although not as efficient as best fungicides where the efficacy is practically 100% (Nielsen 2000). For two reasons efficiacy of hot-water treatment can not be expected to reach 100% as fungicides, a) there is a risk of recontamination, and b) there is a risk for soil borne infection (Borgen 2000). However, in the case of organic farming this uncertainty must be accepted.

The effect of washing observed in experiment 2 is comparable to results found by others (13). Washing and hot-water treatment are not directly comparable in this case, since washing was made in larger amounts of water, including several batches of clean water. This method was chosen to explore the potential of the washing method. Results generally show that the "pure" effect of washing in tap-water reduces the attack by approximately 50% (Figure 2a-e and Figure 1), which is considered a truer picture of the potential for washing than results presented in the second experiment (Table 1).

Conclusively in the fourth and fifth experiments, a reduction in attack was maintained within the treatment window (Figure 3). Harmful effects on both germination percentage and speed of germination were visible only at the highest temperatures. The criteria used to establish the treatment window of course can be discussed, since there are no established standards to be met. Even though those considerations are valid for this experiment only, it is our judgement that the criteria used (i.e. reduction in attack > 95% and reduction in germination properties < 5%) could be considered a rule of thumb in other experiments as well. A higher degree of precision would demand a larger amount of ears to be counted, as was done in experiment one and two, where about 4500 ears per treatment were counted (Table 1).

In previous publications on warm- and hot-water treatment (19, 23, 24, 25) warm-water, i.e. lower temperature (usually <50°C), and longer treatment times (usually >20 minutes) and/or presoaking are investigated and recommended (24, 25). Our results indicate absolutely no necessity for warm-water treatment and/or presoaking, since high efficacy can be reached completely without pre-soaking. Already in the old days, the problems of re-drying was one of the most serious objections against water based thermotherapy (17). As the treatment efficacy is satisfactorily, minimizing re-drying problems and costs is the major challenge. This fact points in favour of hot-water methods with the shortest treatment times. Our study indicate that optimal treatment combinations are at higher temperatures and shorter treatment times than normally considered (Figure 3 and Table 3), i.e. 60°C for 1 minute without any presoaking provided the best overall combination.

These experiments showed a definite potential for the hot-water method, with an effect coming close to current chemical seed treatment. Before this method can eventually be implemented in organic agriculture, large-scale equipment including refinement of treatment protocols needs to be developed. However, we believe this should definitely be possible.

Acknowledgments
Laboratory technician Susane Olsen did careful counting of major part of ears and plants comprising this study, and research assistant Bent Jeppesen gave valuable assistance in treatment and sowing. Agricultural adviser Carsten Markussen supported the development of the idea in the first place and finally senior researcher Kristian Kristensen gave valuable support regarding statistical questions. This study was possible through grants from the Danish Ministry of Food, Agriculture and Fisheries, Strukturdirektoratet. We are grateful for all support.

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