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Reduced Microbiological Contamination Following Irrigation of Germinated Seed for Foods

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Abstract

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Germinated seeds are rich in various nutrients but are vulnerable to fungal contamination which favours micromycete formation on the sprouts. The main aim of this work was an investigation of strategies to reduce the contamination of sprouted seed foods. Over the course of 96 hours of sprouting, seeds of organic spring wheat (*Triticum aestivum* L.), winter wheat (*Triticum aestivum* L.), naked oat (*Avena nuda* L.), triticale *(xTriticosecale*) and rye (*Secale cereale* L.) were irrigated in water filtered using the Pazdroid Med-1500 filtration device with and without 4% ethyl alcohol. Germinated seeds were stored at 18°C for one, three and seven days and the levels of *Mucor* spp., *Penicillium* spp., *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp. and *Bipolaris* spp were determined. Micromycete numbers were greater in sprouted winter wheat and rye but were reduced when these were soaked and irrigated with filtered water and filtered water containing 4% ethyl alcohol. Filtered water led to greater reductions in micromycete numbers in sprouted winter and spring wheat than in other seeds.

Keywords: cereals; filtered water; pathogens; sprouted seeds

Plant-based foods represent a major staple diet for humans in developing countries, where the consumption of animal foods is limited by economic and/or religious concerns; however, such plant-based diets are often associated with micronutrient deficits, exacerbated in part by poor micronutrient bioavailability (Gibson *et al.* 2006). Thus, to improve human health, provision of safer and more nutritious food products is desired. Seed sprouting is a commonly used processing method for seed based foods that effectively improves nutritional quality by increasing the availability of vitamins and micro elements (Blok *et al.* 2000; Swieca *et al.* 2013). Accordingly, sprouts reportedly have up to 100 times the enzyme content of fruits and vegetables. Moreover, during germination, concentrations of vitamins such as vitamin B increase by 4–12 fold after several days of germination (El Adawy *at al.* 2003). Germination is a simple, low-cost process that results in a natural product, eliminates or inactivates certain antinutritional factors (protease inhibitors as Bowman-Birk and Kunitz) and increases legume protein and starch digestibility. Mineral concentrations are also higher during seed germination (Robles-Ramírez *et al.* 2011).

Sprouted seeds are still considered a high-risk food and have been implicated in numerous cases

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of foodborne illness stretching back for more than 30 years (Warriner & Smal 2014). Recent outbreaks of foodborne illness have been linked to the consumption of contaminated sprouts. The spent irrigation water used to irrigate sprouts can carry many microorganisms, including pathogenic strains of *Escherichia coli* and *Salmonella enterica*. These pathogens are believed to originate from the seeds. The U.S. Food and Drug Administration recommends that sprout producers conduct microbiological testing of spent irrigation water from each production lot at least 48 h after seeds have germinated (Kramer & Lim 2004). Much research into food-borne human pathogens has focused on transmission from foods of animal origin. However, recent investigations have identified fruits and vegetables as the source of many disease outbreaks. Now believed to be a much larger contributor to produce-associated outbreaks than previously reported, norovirus outbreaks are commonly caused by the contamination of foods by the hands of infected workers (Berger *et al*. 2010). Sprouted seed foods are grown using various agrotechnical and agrochemical techniques with differing soil types, providing a variety of growth conditions for different microorganisms. Fungal micromycetes that destroy sprouted seed foods can be present in purchased seeds or may be contracted from the environment (Joffe & Palti 1974; Husson 2013). Microorganisms cause do not only cause changes in the sensory quality of sprouted seeds but also synthesise toxic compounds that are harmful for human health (Kordušienė *et al*. 2010). Grains that are processed under outdoor conditions are particularly vulnerable to microorganisms, and their contamination levels are directly correlated with weather conditions during maturity. Common fungal seed contaminants include *Fusarium*, *Alternaria*, *Cladosporium*, *Aspergillus* and *Penicillium* fungi (Harazim *et al.* 1998; Sweeney & Dobson 1998; Trevor & Suslow 2004), which can be distributed by wind, water, insects and birds.

Fresh produce is frequently contaminated by microorganisms, which may lead to spoilage or even pose a threat to human health. Sprouts are considered to be among the most risky foods sold at retail since they are grown in an environment practically ideal for growth of bacteria and are usually consumed raw (Butscher *et al.* 2016). While the role of seeds as a primary inoculum source in plant disease outbreaks is well-established, the significance of seedborne inoculum for human pathogens has been only recently recognised. In many of these cases, vegetable seed sprouts have been implicated as the initial inoculum source. Raw seeds are currently considered the most significant source of inoculum for foodborne illnesses associated with sprout consumption (Buck *et al*. 2003). Most organisms grow slowly on dry seeds, and the presence of sufficient moisture strongly influences important plant physiological processes (Husson 2013). The World Health Organization estimated that 80% of diseases on the planet are associated with the use of contaminated water, and various physical methods and filtration devices are widely used to improve water quality; however, drinking water is commonly used to germinate seeds, potentially leading to contamination. Berdishev and Novichenko investigated the physical, chemical and biological properties of snow water from far northern latitudes and associated 20–35% reductions in deuterium and tritium levels with improved agrochemical properties (Berdishev & Novichenko 2009). Moreover, popular water treatment technologies are widely used to produce ionised water with improved properties. Specifically, reduction of deuterium and tritium isotope contents in water led to improved germination of wheat, as reflected by larger seedling lengths and sizes (Petrus-Vancea *et al.* 2010).

Moisture is sufficient for the emergence of micromycetes, and sprouted seeds are produced primarily by soaking in water followed by heat sprouting, providing conditions that are highly favourable for the development of microorganisms.

In addition, mature seeds lack strong protective layers and chemical defences against microbial damage (Trevor & Suslow 2004), and uneven distributions of moisture in grain masses are an important factor for micromycete development (Swieca *et al.* 2013). Herein, we investigated the effects of filtered water on irrigation quality indicators and mycological contamination of sprouted seeds for food.

MATERIAL AND METHODS

All experiments were performed during the years 2015 and 2016 at the Institute of Agriculture and Food Sciences of the Agronomy Faculty of Aleksandras Stulginskis University, Lithuania. Seeds of spring wheat (*Triticum aestivum* L. cv. Rospuda), winter wheat (*Triticum aestivum* L. cv. Zentos), naked spring oat (*Avena nuda* L. cv. Dinaro), winter rye (*Secale cereale* L. cv. Mina DS) and winter triticale

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(× *Triticosecale* Wittmack cv. Virgiai) were obtained from an organic farmer. Seeds were germinated in 1-L Vilmorin multipliers (Vilmorin Garden, CNOS PNO Sp. z o.o., Poland) of 20 cm in diameter that had been disinfected with ethyl alcohol. Multipliers comprised an excess water collector, three germination bowls and a cover, and sprouting bowls had wavy bottoms and a siphon drain to remove excess water. Seeds were carefully selected, and damaged, fragmented and discoloured seeds were removed. Selected seeds were then soaked for 12 h in four volumes of drinking water, filtered water or filtered water containing 4% ethyl alcohol (seed:water ratio, 1 : 4). After 12 h, the water was drained from the seeds using a percolator until the seeds were moist but not wet. Seeds were sprouted for 96 h at 23–25°C in a dark ventilated room. Germination procedures were performed four times with 50 g of seeds. For identification of pathogen content, the germinated seeds were stored at 18°C for one, three and seven days (here after referred to as storage).

The drinking water quality indicators that determine the different quality parameters of water used for seed soaking and irrigation are presented in Table 1. Drinking water was filtered through a Pazdroid Med-1500 (Efkotas, Lithuania) filtration unit that uses the electromagnetic molecular dissociation principle. The filtration device is a new invention and its details are withheld due to a patent application; however, following filtration through the device, water has a negative redox potential (–60 to –70) and 30–35% lower concentrations of the hydrogen isotopes deuterium and tritium (Table 1).

During the germination process, sprouted seeds were irrigated using multipliers every 24 h in 500 ml of drinking water, filtered water or filtered water containing 4% ethyl alcohol. Ethyl alcohol was used as a disinfectant and the treatment solution was prepared by adding 40 ml of ethyl alcohol 98% percent to 1 l of filtered water. The effects of alcohol on seed germination and dormancy depend on alcohol concentration, duration of the treatment and plant species. Longer exposure may kill the seed, but in some cases, alcohol can break the seed dormancy. A low concentration is effective for dormancy breaking. Alcohol acts on cell membranes, but can also dissolve and wash out inhibitors that may be present in the seed coat (Taylorson & Hendricks 1979). Dao and Dantigny (2011) reviewed the effects of ethanol on growth and germination of fungi and on the inactivation of fungal spores. Following survey of the literature, they concluded that sensitivity to ethanol is highly dependent on the mould. Minimum inhibitory concentrations of ethanol are about 4.2% for *Aspergillus niger* and 3.93% for *Penicillium chrysogenum* (Dao & Dantigny 2011).

After 96 h, sprouting seeds were weighed and 60% and 40% portions were used to determine the chemical composition and microbiological contamination, respectively. Comparisons were made between the grain seed species spring wheat, winter wheat, naked oats, triticale, and rye, following irrigation with drinking water (control), filtered drinking water and filtered drinking water containing 4% ethyl alcohol.

For the identification of the pathogens in germinated seeds during storage, 10-g samples of sprouts

Table 1. Water quality indicators

No.	Parameter (unit)	Water quality standards; *HN	Drinking water	Filtered drinking water	Filtered drinking water containing 4% ethyl alcohol
1	pH	6.5–9.5	7.29	7.55	7.44
2	ammonium, NH_4^+ (mg/l)	0.5	0.47	0.31	0.22
3	nitrites, NO_2^- (mg/l)	0.1	0.05	0.01	0.02
4	nitrates, NO_3^- (mg/l)	50	3.0	1.0	1.0
5	phosphates, PO_4^{-3} (mg/l)	–	0.1	–	–
6	O_2 (mg/l)	–	9.81	5.2	7.2
7	hardness	–	32.5	29.4	29.4
8	iron, Fe^{++} (mg/l)	0.2	0.27	0.18	0.22
9	permanganate index (mg O_2/l)	5	16.4	7.2	8.8
10	specific electric conductivity (μS/cm) 20°C	2500	550	190	160
11	redox potential		241	149	44

*HN – hygiene standard (24:2003)

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were placed in 90-ml aliquots of sterile water and were then diluted between four and ten times. Final dilutions were then added to petri dishes containing malt extract agar and were incubated for three days at 24°C; colony forming units were then counted (Joffe & Palti 1974).

The significance of the differences between the means was determined according to the least significant difference (LSD) at 0.01 probability level. The data were processed using the DISVEG software from the Selekcija package.

RESULTS AND DISCUSSION

Sprouted food seeds are generally consumed raw, necessitating high product quality and safety; however, washing does not always remove all bacteria and micromycetes from commercial seed products (Kordusiene *et al*. 2010). In particular, fungal microbes are widely detected on grains, and it is important to identify amounts and species compositions of such contaminants so that the processing steps at which they are introduced can be identified (Eriksen & Aleksander 1998).

A previous study performed by Frazier and Westhoff (1988) showed that micromycete-contaminated foods are not always toxic; however, fungal mycotoxins are persistent and can remain in foods long after the fungi have been eliminated. A limited survey of fresh and minimally processed vegetables and sprouts was conducted in the Washington, D.C. area to determine if potentially toxigenic and pathogenic fungi were present in these commodities. Thirty-nine ready-to-eat salads, 29 whole fresh vegetables and 116 sprout samples (bean, alfalfa, broccoli, crunchy, garlic, spicy, onion, clover, lentil and multi-seed sprouts) were purchased from 13 local supermarkets and tested for yeast and mould counts as well as for the presence of toxigenic moulds. The most common moulds found in fresh and minimally processed vegetables were *Cladosporium*, *Alternaria* and *Penicillium*; less common was *Geotrichum*. The most frequently isolated moulds from sprouts were *Alternaria*, *Cladosporium*, *Penicillium*, and *Phoma* (Tournas 2005). Thus, to avoid contamination of food grains with mycotoxins, micromycete development must be prevented by disinfection. In recent studies, toxic micromycete compounds such as trichothecenes and aflatoxins were shown to be particularly pathogenic in humans. Moreover, extensive studies of micromycete species diversity have identified factors that contribute to their spread, resulting in damage to food sources (Kordusiene *et al*. 2010).

In this study, we determined the levels of *Mucor* spp., *Penicillium* spp., *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., and *Bipolaris* spp. micromycetes in seeds immediately after 96 h of germination and after one, three and seven days of storage at 18°C. Sprouted seeds mostly contained *Penicillium* spp., *Alternaria* spp., *Aspergillus* spp., and *Fusarium* spp. (Figure 1A).

As mentioned above, the aim of this study was to investigate the effect of water, so only one species of sprouted seeds was investigated with unsprouted seeds. Unsprouted spring wheat seeds were compared with sprouted spring wheats. After seven days of storage of spring wheat sprouts that were irrigated with drinking water, we mainly observed contamination with *Alternaria* spp. (32%) and in unsprouted seeds only (8.2%). *Fusarium* spp. contamination was found in 26% of sprouted seeds and in 23.4% of unsprouted spring wheats seeds. *Aspergillus* spp. micromycetes were found in 16.42% of unsprouted seeds, these were not detected in sprouted seeds. *Penicillium* spp. micromycetes were detected in only 26% of unsprouted seed samples and in 9.24% of sprouted spring wheats (Figure 1A).

After seven days of storage of spring wheat sprouts that were irrigated with drinking water, we mainly observed contamination with *Fusarium* spp. (23.08%); following irrigation with filtered water and filtered water containing 4% ethyl alcohol, *Fusarium* spp. contamination was identified in only 15.38 and 7.69% of sprouted seed samples, respectively, however. These data show that filtered water can be used to reduce contamination of sprouted seed foods. In a previous study, Heintze (2009) correlated changes in bacterial contaminants with redox potential. In agreement with his results, the filtered water used in this study had a lower redox potential than drinking water and its use for irrigation resulted in decreased numbers of micromycetes.

Spring wheat sprouts that were irrigated with drinking water contained no *Fusarium* spp. micromycetes or *Aspergillus* spp. after one and three days of storage; *Penicillium* spp. micromycetes were detected in only 9.62% of samples. Irrigation with filtered water reduced this contamination rate to 1.92%.

Sprouted winter wheat seeds had almost twice the numbers of micromycetes of sprouted spring wheat seeds (Figure 1B). Moreover, very few *Penicillium*

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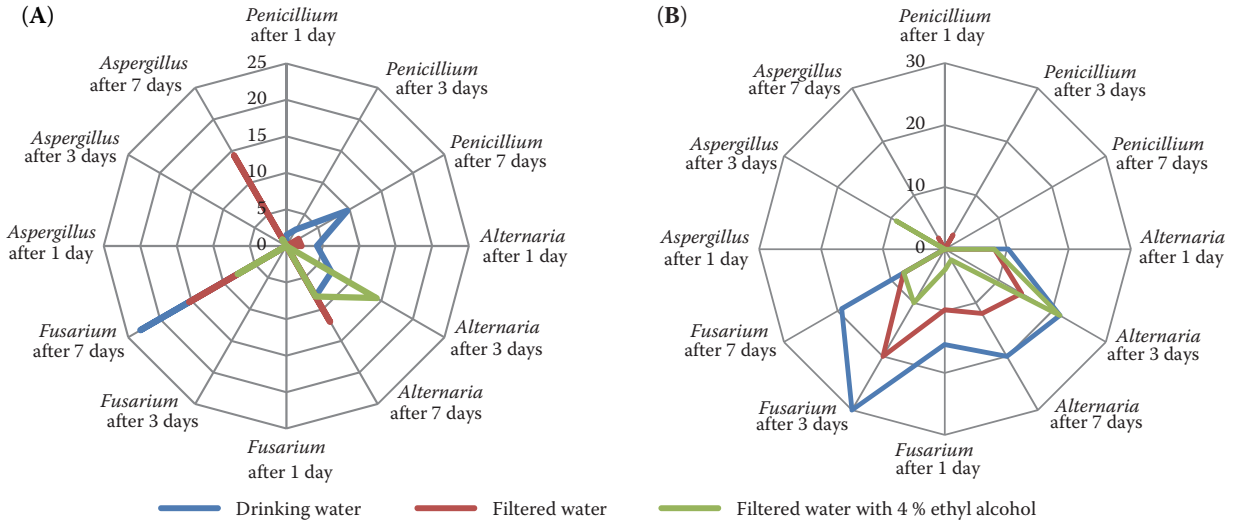

Figure 1. Percentage frequencies of micromycetes in sprouted (**A**) spring wheat and (**B**) winter wheat seeds

The microbiological evaluation: after 1, 3, and 7 days of storage of spring wheat sprouts; 0–30 – detection rate of different fungi tribes (%)

spp. micromycetes were detected in sprouted winter wheat seeds (2.6%), although *Aspergillus* spp. micromycetes were slightly more numerous and *Alternaria* spp. and *Fusarium* spp. were dominant. Specifically, *Alternaria* spp. were present in 21.43% ($P < 0.01$) of seed samples that were irrigated with drinking water for three days of storage; however, these species were present in only 14.29% of samples after irrigation with filtered water. Irrigation with filtered water containing 4% ethyl alcohol resulted in similar rates of contamination as in drinking water controls (21.43%). In contrast, *Fusarium* micromycete frequencies in winter wheat seeds were decreased by irrigation with filtered water either with or without 4% ethyl alcohol. Pathogens such as *Fusarium* have previously been controlled by lowering the redox potential of irrigation water (Blok *et al.* 2000).

Fusarium spp. micromycetes were abundant in naked oats (Figure 2A), with maximum detection rates of 20% after three days of storage of sprouts that were irrigated with drinking water. Takehara *et al.* (2004) controlled soil-borne pathogens by reducing

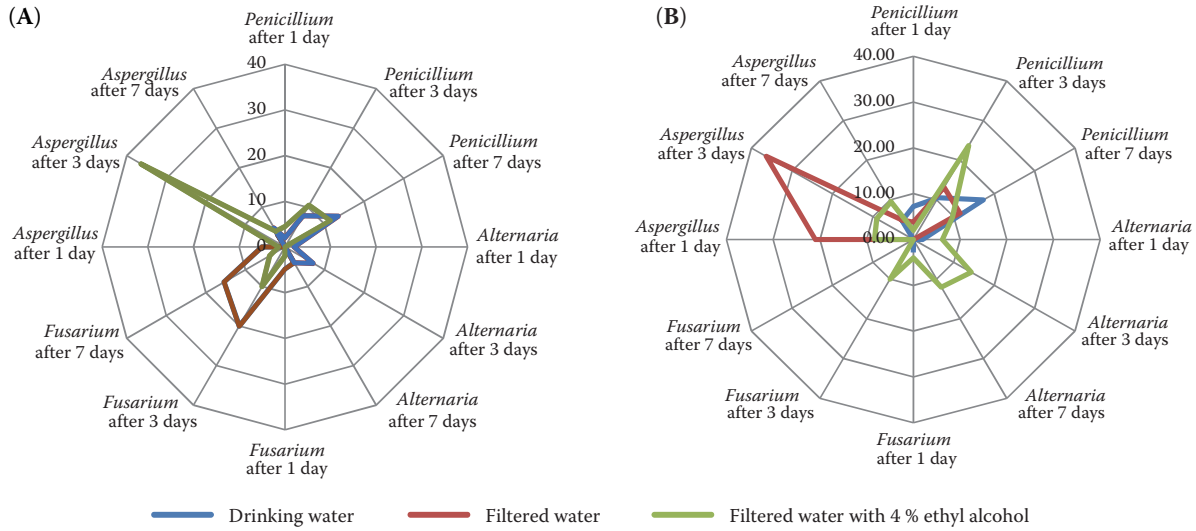

Figure 2. Percentage frequencies of micromycetes in sprouted (**A**) naked oats and (**B**) rye seeds

The microbiological evaluation: after 1, 3, and 7 days of storage of spring wheat sprouts; 0–30 for (A) and 0–40 for (B) – detection rate of different fungi tribes (%)

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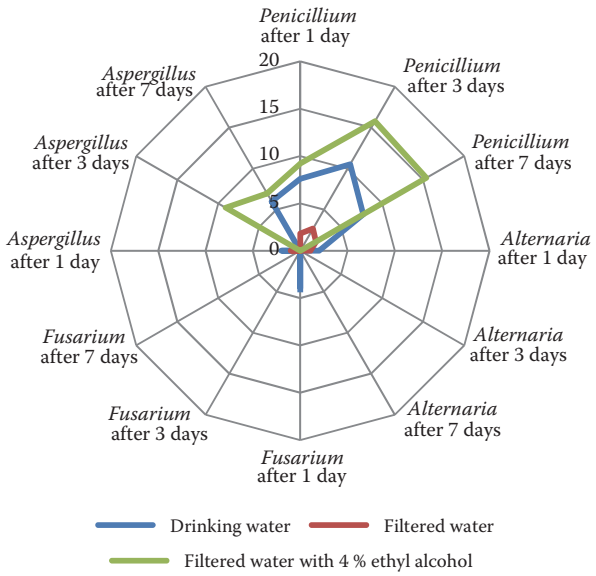

Figure 3. Percentage frequencies of micromycetes in sprouted triticale seeds

The microbiological evaluation: after 1, 3, and 7 days of storage of spring wheat sprouts; 0–20 – the detection rate of different fungi tribes (%)

soil redox potential and the present data confirmed these observations in seeds sprouted in water of low redox potential. When comparing all fungal species in naked oats, *Aspergillus* was dominant (36.36%) ($P < 0.01$), and the other species were detected at rates of 2–16%.

Micromycetes were detected in sprouted rye seeds at similar frequencies to those in winter wheat seeds (Figure 2B). Irrigation with filtered drinking water was most effective against *Penicillium*, decreasing micromycete numbers from 7.2% to 2% immediately after germination, and from 17.31% to 9.62% after seven days of storage, respectively. *Fusarium* and *Alternaria* micromycetes were not detected following irrigation with drinking water or filtered water for three and seven days of storage; however, increased numbers of *Aspergillus* micromycetes were observed in sprouted rye seeds after irrigation with filtered water in the presence and absence of 4% ethyl alcohol.

Micromycetes were observed in triticale seeds at very low rates (Figure 3). In these experiments, *Alternaria* and *Fusarium* micromycetes were detected in only 1–4% of samples immediately after germination, and no micromycetes were detected after three and seven days of storage; however, *Penicillium* spp. micromycetes were detected in 15.79% ($P < 0.01$) of triticale samples.

CONCLUSIONS

Fusarium spp. micromycetes were dominant in sprouted winter wheat seeds at germination times of one, three and seven days of storage; however, *Penicillium* dominated in triticale and rye after three days of storage, *Aspergillus* dominated in naked oats and *Alternaria* dominated in spring wheat. After seven days of storage, *Penicillium* contamination was maximal in naked oats, rye and triticale and *Fusarium* micromycete numbers were maximal in spring wheat.

Irrigation with filtered water led to decreased micromycete numbers in all types of sprouted seeds, particularly in spring and winter wheat, with 80, 35, and 50% decreases in *Penicillium*, *Fusarium*, and *Alternaria* micromycete numbers in spring wheat, respectively, and 33–66% reductions in *Alternaria* and *Fusarium* micromycete numbers in winter wheat samples.

Filtered drinking water containing 4% ethyl alcohol reduced microbiological contaminations in all species of seeds, particularly in sprouted spring and winter wheat seeds. Finally, *Alternaria* and *Fusarium* micromycetes were completely eradicated by irrigation of spring wheat with filtered water, and were decreased by 21 and 90% in sprouted winter wheat seeds, respectively.

The type of drinking water had a significant effect on the decrease of micromycetes. Filtered drinking water and filtered drinking containing 4% ethyl alcohol exhibited different quality indicators to those of drinking water.

In conclusion, filtered drinking water and filtered drinking water containing 4% ethyl alcohol are effective means to slow the development of micromycetes, which are potential producers of mycotoxin. Employing these waters would be a simple, efficient and inexpensive way to reduce the microbiological contamination of sprouted seeds.

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