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Liquid nitrogen controls seed-borne chalcids without reducing germination in coriander seeds

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Abstract
Coriander seeds are susceptible to infestation by chalcid wasps which often render the seeds inviable. Control of chalcids in seeds is a prerequisite for supplying coriander germplasm to requestors throughout the world. Levels of chalcid infestation in coriander seed samples produced at the North Central Regional Plant Introduction Station, in Ames, IA, mandated the need to develop an effective control strategy without harming the seeds. Storing the seeds above liquid nitrogen for 16 hours proved effective in killing chalcids at all life stages without reducing seed germination. Results were based on germination tests, seed dissection, chalcid emergence, and digital x-ray images.

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Summary

Coriander seeds are susceptible to infestation by chalcid wasps which often render the seeds inviable. Control of chalcids in seeds is a prerequisite for supplying coriander germplasm to requestors throughout the world. Levels of chalcid infestation in coriander seed samples produced at the North Central Regional Plant Introduction Station, in Ames, IA, mandated the need to develop an effective control strategy without harming the seeds. Storing the seeds above liquid nitrogen for 16 hours proved effective in killing chalcids at all life stages without reducing seed germination. Results were based on germination tests, seed dissection, chalcid emergence, and digital x-ray images.

Introduction

Coriander (*Coriandrum sativum* L.) is an annual member of the umbel family (Apiaceae) grown for its aromatic fruits and leaves, which are used to flavor foods and in cosmetics (Weiss, 2002). More than 200 coriander germplasm accessions are conserved by the North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa as part of the U.S. National Plant Germplasm System, a network of genebanks coordinated by the United States Department of Agriculture – Agricultural Research Service. This collection preserves a diverse array of forms (Lopez and Widrlechner, 2004), which are widely distributed for use in scientific research and crop improvement. Periodic regenerations of coriander germplasm are conducted at the NCRPIS to replenish seed supplies.

Coriander seeds are susceptible to infestation by chalcid wasps, *Systole coriandri* (Gussakovsky) and/or *S. albipennis* (Walker). Female wasps lay eggs in young fruits, and larvae subsequently feed on and pupate in the developing seeds. Damaged seeds are often inviable. But there is an additional problem: the possibility that, as coriander germplasm is distributed to meet users' needs, chalcids may be transported to regions where they had not been found previously.

Lamborot *et al.* (1993) reported that seed damage is done very early after egg deposition, which is generally not possible to observe. Pupae and final-instar larvae typically remain dormant (in diapause) in the seeds over winter. Some individuals complete their life cycle within a single growing season while others remain in diapause for more than one winter.

Once diapause is complete, the adult emerges from the seed by creating a small exit hole in the seed coat, which can be easily overlooked (figure 1A). An examination of coriander seeds regenerated at the NCRPIS revealed a local chalcid infestation by 1992. Chalcid specimens from recently infested coriander seeds were sent to the Systematic Entomology Laboratory, Beltsville, Maryland and identified as *S. coriandri* (Gates, personal communication).

Figure 1. Digital images of coriander seeds. A. Intact seeds with chalcid exit holes, indicated by arrows. B. Live larvae from controls (left two) and dead larvae from LN_2 treated coriander seeds (right two). C. Dead larvae from 'natural' causes compared to LN_2 treated larvae. Top row 'natural', bottom row LN_2. Magnification levels of images are not equal. Size scales are in millimeters.

Lamborot *et al.* (1993) reported that chalcid infestations were first detected in Chile in 1984, but by 1986, infestation levels at some locations were as high as 75%. They surmised that the introduction of *S. coriandri* resulted from importation of infested coriander seeds from Europe and Asia, and attributed its rapid dispersal in Chile to the lack of detection in infested commercial seed and inappropriate cultural practices.

Several methods have been described to eliminate chalcid infestations in coriander seeds. Verma (1986, 1987, 1988) reported effective control of chalcids in coriander seeds by fumigating with methyl bromide and hydrocyanic acid without deleterious effects on seed viability. However, the complexity of the treatment apparatus, the unusual method of soaking seeds before treatment as reported in one paper (Verma, 1988), and the inherent danger to personnel by exposure to toxic gases rendered these methods unsuitable.

Several researchers have used modified atmospheres (N_2 and CO_2) to control seed-borne larvae of red flour beetles (*Tribolium castaneum* Herbst) and almond moths (*Ephestia cautella* (Walker)) (Donahaye *et al.,* 1996; Jay and Cuff, 1981; Navarro and Calderon, 1973). These researchers concluded that the modified atmospheres killed larvae via desiccation. However, these protocols also require sophisticated apparatus and expose seeds to relatively high temperatures for durations that may decrease long-term viability.

The application of low-temperature treatment is more promising. As noted by Strang (1999), thermal methods require no toxins for initial treatment nor do they produce toxic residues, and they are widely available. Large quantities of seed can be treated quickly at relatively low cost. Richards and Hanna (1982) and Richards (1989) found that exposing chalcid-infested sainfoin (*Onobrychis viciaefolia* Scop.) seeds to a five-day treatment at -40 °C effectively destroyed larvae of the sainfoin seed chalcid (*Eurytoma onobrychis* Nikolskaya) without harming seed viability. The use of even lower temperatures from liquid

nitrogen (LN_2) to kill wood-destroying insects has been reported by several researchers (Lewis and Haverty, 1996; Rust *et al.,* 1997; Taylor *et al.,* 1997). The effectiveness of LN_2 treatments was quite high in some situations, although structural damage to the test objects, such as board and tiles, was often encountered. Fortunately, coriander seeds are regularly stored above LN_2 (i.e., in the vapor phase) without significant loss of viability at the National Center for Genetic Resources Preservation in Fort Collins, Colorado (Miller, 2003, personal communication). Thus, experiments were initiated to test the effectiveness of LN_2 in destroying chalcid larvae in coriander seeds and measure its effects on seed viability.

Material and methods

Seven accessions of coriander (table 1) regenerated under field conditions at the NCRPIS in 2001 were evaluated. Accessions were planted in April and harvested in July and August. After the final harvest, these seeds were stored at approximately 18°C and 50% Relative Humidity (RH) for three months and then at 4°C (40% RH) for five months before testing. The following year, eight additional accessions (table 1) were regenerated for testing. These were planted in May and harvested in August and September. After the final harvest, these seeds were stored at approximately 18°C (50% RH) for one month and then at 4°C (40% RH) for seven months before testing. Differences in the duration of storage at 18°C and 4°C between years were due to attempts to simulate different chalcid-overwintering regimens based on Strong (1962). Neither method proved adequate.

Table 1. Coriander accessions used in the 2002 and 2003 studies.

Accession	Origin	Chalcid Infestion (%)
2002 Study (2001 harvest)		
Ames 13899	Tajikistan	–
Ames 18583	France, Seine-Maritime	16
Ames 20048	Armenia	–
Ames 21655	Russian Federation	22
Ames 23638	Oman	–
Ames 23641	Oman	–
Ames 24917	Portugal	–
2003 Study (2002 harvest)		
Ames 18594	United Kingdom, England	21
Ames 20046	Azerbaijan	32
Ames 24909	United States	28
Ames 24915	United States	20
Ames 24927	Former Soviet Union	44
Ames 25170	United States, Iowa	15
Ames 26829	Mexico, Puebla	31
PI 174130	Turkey	21

– not determined

Percent infestation was determined by seed dissection, accomplished by using a dissecting microscope, fiber-optic light, and dissecting implement. Fruits were first split in half by pressing on them while they lay in a watch glass. A probe or dissecting needle was used to separate the two halves of each fruit and to slit the internal membrane which covers the contents of the seed. The contents of the seed were recorded as: good seed embryo, empty or underdeveloped embryo, exit hole (wasp already left seed), dead larva (denoted by brown coloring or some desiccation), live larva (remain white in color and "plump" shape), pupa, or adult. Percent infestation was calculated by totaling the number of seeds with holes, dead and live larvae, pupae, and adults and dividing by the number of seeds examined.

LN_2 treatments consisted of placing 50 fruits (100 seeds) per replication in small paper coin envelopes, which were then placed in 13.3 × 13.3 × 5.2 cm polycarbonate cryogenic storage boxes. These boxes were placed over LN_2 at approximately -156°C for 24 hours in 2002 and 16 hours in 2003. After removal from the cold treatment, the seeds in their coin envelopes were immediately placed in impermeable plastic bags to limit moisture uptake by the seeds due to condensation.

Tests were conducted for seed-moisture content, seed germination, and chalcid emergence for both treated samples and untreated controls. Moisture content was determined by the low constant-temperature oven method as described in the International Rules for Seed Testing (International Seed Testing Association, 1993) for oilseed crops. Our measurements consisted of weighing three replications of 50 fruits (100 seeds) before and after oven drying at 103°C for 17 hours. For the 2002 study (2001 harvest), some seed quantities were limited. In such cases, moisture content was based on only one or two replicates.

Germination tests consisted of imbibing three replications of 50 fruits (100 seeds) in 12.5 × 13 × 3.5 cm plastic boxes containing two germination blotter papers wetted initially with 27 ml of water. The germination regimen consisted of 8 hours at 30°C with fluorescent lights alternating with 16 hours at 20°C in the dark. The germination tests ran 28 days for the 2002 study and 42 days for the 2003 study, in an attempt to obtain more accurate and complete results. Because of the low germination levels observed in the 2003 study, a follow-up germination test was conducted in 2005 to attempt to account for the low germination rates previously observed.

A chalcid emergence test was conducted by placing 50 fruits (100 seeds) in 450 ml glass jars with screw-top lids, replicated three times. The jars were placed in walk-in incubators set at 15°C for 20 weeks and monitored monthly to record how many chalcids emerged from the coriander seeds. Because chalcid emergence was low for all treatments, a follow-up study was conducted to confirm the effectiveness of the LN_2 treatment. Three accessions of coriander seed (table 5) harvested in 2004 (two replications of 50 fruits each) were subjected to a 16-hour treatment above LN_2 and then stored at room temperature for two and four months to allow for the visible evidence of tissue decay (i.e. browning reaction) that corresponds to evidence for dead larvae. Whether the browning reaction caused by the LN_2 treatment was enzymatic or non-enzymatic was not determined. According to Nursten (2005), this is very difficult to judge. Determinations of live versus dead larvae were made by dissecting the seeds as described above. Untreated

controls (two replications of 50 fruits each) were also examined and compared to the LN_2 treated seed.

Due to the tedious nature of seed dissection, in 2005, X-ray imagery (Tay *et al.,* 2005) was tested as a tool to help quantify chalcid infestation. Two replicates of 50 fruits (100 seeds) each of the LN_2 treated and untreated controls of three accessions of coriander (table 5) were individually placed in wells of 96-well, clear-bottom plastic assay plates. The fruits were kept in place by covering the assay plates with clear-plastic laminate material taped at the edges. The fruits were X-rayed by using a Faxitron MX-20 apparatus (Faxitron X-Ray Corporation, Wheeling, IL 60090 USA) with a non-adjustable, polychromatic beam (Wigman, 2005, Faxitron X-Ray Corporation, personal communication). Exposure time was 20 seconds at 20 KV. The X-ray images were captured digitally and used to quantify seed infestation and help determine chalcid mortality. After the images were produced, the seeds from the assay plates were dissected and inspected for comparison to measurements made from the images.

Statistical analyses were conducted by using SAS software (SAS Institute Inc., 1989). All comparisons of means were made on the basis of Student's t-test.

Results and discussion

Fifteen coriander accessions were tested in the 2002 and 2003 studies, representing germplasm from the New and Old Worlds (table 1). Chalcid infestation levels ranged from 15 to 44% (table 1), highlighting the need to eliminate chalcids before the seed is distributed nationally or internationally. Percentages reported in this paper are based on the total number of seeds not on the number of fruits.

Seed moisture for the samples tested in 2002 and 2003 varied from 7.4 to 12.9% before LN_2 treatment and from 7.3 to 10.2% after treatment (table 2). In a study by Vertucci (1989), seeds of pea, soybean, and sunflower could be stored over LN_2 at an intermediate range of moisture content (8 to 18%, dry weight) without deleterious effects to viability when cooling rates of 1 to 200°C/min were utilized. The method employed in our experiments fell within the cooling rates described by Vertucci (1989) and is similar to the method used at the National Center for Genetic Resources Preservation for storing coriander seeds.

For the most part, seed germination was not affected by LN_2 treatments (table 3). Germination of samples tested in 2002 varied from 18 to 96% before LN_2 treatment and from 11 to 98% after treatment, with only one of the seven accessions displaying a significant decrease in germination after treatment (table 3). Germination for samples tested in 2003 was poor, ranging from 5 to 23% before LN_2 treatment and from 6 to 27% after treatment. Of the eight accessions, one (Ames 18594) displayed a significant increase in germination and another (Ames 20046) displayed a significant decrease. A follow-up germination study conducted in 2005 (data not shown) showed no improvement in percent germination over the 2003 results; discounting the possibility that initial dormancy and the need for after-ripening was a factor. A spot-check (i.e. dissections of one replicate of 100 seed per accession) of eight accessions grown in 2001 (two of which were used

Table 2. Effect of treatments on seed moisture content (%, dry weight basis).

Accession	Control	After LN_2 treatment[1]	Significance level[2]
2002 Study (2001 harvest)			
Ames 13899	7.4	7.3	ns
Ames 18583	9.3	9.7	ns
Ames 20048	9.1	9.7	0.05
Ames 21655	12.9	10.2	0.08
Ames 23638	7.6	7.8	ns
Ames 23641	7.7	7.6	ns
Ames 24917	7.7	7.5	ns
2003 Study (2002 harvest)			
Ames 18594	9.4	9.9	ns
Ames 20046	9.6	9.8	ns
Ames 24909	9.3	9.8	ns
Ames 24915	9.5	9.1	ns
Ames 24927	10.2	10.0	ns
Ames 25170	8.4	8.6	ns
Ames 26829	9.3	9.1	ns
PI 174130	9.0	8.3	ns

[1] The 2002 study received a 24 hr treatment. The 2003 study received a 16 hr treatment
[2] ns = no significant difference.

Table 3. Effect of treatments on seed germination (%).

Accession	Control	After LN_2 treatment[1]	Significance level[2]
2002 Study (2001 harvest)			
Ames 13899	96	98	ns
Ames 18583	18	11	ns
Ames 20048	74	72	ns
Ames 21655	52	50	ns
Ames 23638	92	78	0.05
Ames 23641	66	62	ns
Ames 24917	68	72	ns
2003 Study (2002 harvest)			
Ames 18594	8	12	0.05
Ames 20046	21	15	0.05
Ames 24909	23	27	ns
Ames 24915	11	11	ns
Ames 24927	11	13	ns
Ames 25170	5	6	ns
Ames 26829	19	16	ns
PI 174130	11	19	ns

[1] The 2002 study received a 24 hr treatment. The 2003 study received a 16 hr treatment
[2] ns = no significant difference.

in our 2002 study) and 18 eighteen accessions grown in 2002 (eight of which were used in our 2003 study) revealed differences in the number of empty seeds and chalcid infestation. The 2001-grown seed had an average of 8% empty seed and 15% seed infestation across the eight accessions checked. The 2002-grown seed had an average of 25% empty seed and 23% seed infestation across the eighteen accessions checked. The high proportion of empty seed was confirmed by examining ungerminated seeds after germination testing in 2005 (data not shown). The average germination of these accessions, when based on the number of germinated and ungerminated but filled seeds remaining after the germination test, was 55%, which is more in line with the results of the 2002 study (table 3). The 2001 harvested seeds were planted in mid-April of 2001. The 2002 harvested seeds were planted in early to mid-May, suggesting that coriander is more susceptible to chalcid infestation and poorer seed-fill when planted later in the season. Research specifically focused on planting date could be conducted to test this hypothesis.

Chalcid emergence was completely controlled by the LN_2 treatment (table 4). No chalcids emerged from any LN_2 treated seeds in either the 2002 or 2003 studies. However, chalcids did emerge from five of the seven untreated seed samples tested in 2002 and from six of eight untreated seed samples in 2003. These results imply that the chalcids were completely destroyed by the LN_2 treatment, yet lack of observed emergence in our tests might not equate with complete mortality.

Table 4. Effect of treatments on chalcid emergence (%).

Accession	Control	After LN_2 treatment[1]	Significance level[2]
2002 Study (2001 harvest)[3]			
Ames 13899	0	0	ns
Ames 18583	4	0	0.05
Ames 20048	3	0	0.05
Ames 21655	3	0	0.05
Ames 23638	1	0	ns
Ames 23641	0	0	ns
Ames 24917	1	0	ns
Mean	1.4	0	0.05
2003 Study (2002 harvest)[4]			
Ames 18594	2	0	0.05
Ames 20046	0	0	ns
Ames 24909	1	0	ns
Ames 24915	2	0	0.05
Ames 24927	1	0	ns
Ames 25170	1	0	ns
Ames 26829	0	0	ns
PI 174130	1	0	ns
Mean	0.9	0	0.05

[1] The 2002 study received a 24 hr treatment. The 2003 study received a 16 hr treatment

[2] ns = no significant difference.

[3] Emergence test lasted 141 days.

[4] Emergence test lasted 143 days.

In order to verify the hypothesis that a 16-hour LN_2 treatment completely kills chalcids, a follow-up study (table 5) was conducted. Of the three accessions examined, only one (Ames 23623) had high enough levels of chalcid infestation to allow for a meaningful comparison between treatments. Hand dissections of untreated and LN_2 treated seeds verified that LN_2 treatment destroyed all the seedborne chalcids (table 5, Survival %). Among the four replicates of Ames 23623 dissected, many larvae were alive in the control, although a few larvae had died due to 'natural' causes. No chalcid larvae survived the LN_2 treatment. Figure 1B illustrates differences in color and size between live larvae from the untreated controls and dead larvae from the LN_2 treated seeds. These dissections and images were taken two to four months after treatment in LN_2. Live larvae were white with a visible tan 'gut' running longitudinally. The LN_2 treated larvae turned a translucent brown color but did not shrink in size. This can be contrasted to the color and shape of larvae that died in the seed for non-LN_2 treatment reasons (figure 1C). Those larvae were a golden color or were quite shriveled if they had turned dark brown.

Results of estimating chalcid infestation of coriander by using X-ray imaging were mixed. When an estimate was made for the entire assay plate, the correspondence between estimated and actual infestation levels was very good (table 5, Chalcid Infestation %). No significant differences were found between actual and estimated percentages. However, when each fruit was assessed for chalcid infestation, X-ray imaging was only 79% accurate for Ames 23623 (the highly infested lot) when compared to actual infestation as determined by hand dissections of individual seeds. Whether this level of accuracy is acceptable should

Table 5. Effect of treatments on chalcid survival[1] (%) for 2005 study.

Accession (2004 harvest)	Origin	Chalcid Infestion (%)			
		Actual	Estimated (x-ray)	Significance level[2]	Fruit-by-Fruit[3] Accuracy (%)
Ames 23623	Syria	48	44	ns	79
Ames 26818	Mexico, Pueblo	2	2	ns	89
Ames 26827	Mexico, Pueblo	3	2	ns	88
		Survival (%)			
		Control[4]	After LN_2 treatment[5]	Significance level[2]	
Ames 23623	Syria	27	0	0.05	
Ames 26818	Mexico, Pueblo	0	0	ns	
Ames 26827	Mexico, Pueblo	1	0	ns	

[1] Actual chalcid infestation and survival determined by seed dissection and visual inspection. Estimated infestation was based on x-ray photography.

[2] ns = no significant difference..

[3] Fruit-by-fruit accuracy describes the accuracy when comparing x-ray estimate versus actual dissection for each fruit based on location in the assay plates.

[4] Some replicates of Ames 26818 and 26827 did not have any chalcid infestation and therefore did not allow for significant differences in the statistical analysis.

[5] 16 hr treatment

be determined on a case-by-case basis. Difficulties in determining the percent infestation via X-ray imaging are due to several factors. The double-seed arrangement of coriander fruits is one factor. When fruits displayed the edges of the adjoining seeds (figure 2, end view, e.g. cell 33), an accurate determination of infestation could be made. With other orientations, the top seed sometimes obscured the contents of the bottom seed (figure 2, side view, e.g. Cell 34). Splitting each fruit into two separate seeds may alleviate this problem, but is time consuming, which reduces some of the efficiency of our method.

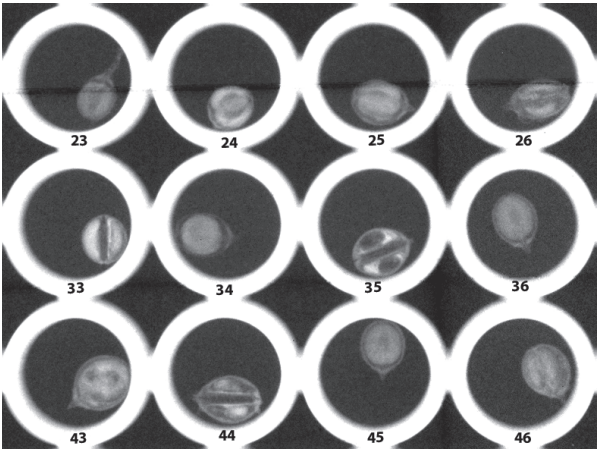

Figure 2. Digital x-ray image of coriander seeds in 12 cells of an assay plate. Hand dissections of the seeds revealed the contents as:

Cell No.	Fruit position	Contents of left or top half of fruit	Contents of right or bottom half of fruit
23	Side[1]	Empty seed	Filled seed
24	Angled[2]	Filled seed	Filled seed
25	Angled	Filled seed	Filled seed
26	End[3]	Chalcid	Live larva
33	End	Filled seed	Live larva
34	Side	Filled seed	Filled seed
35	End	Seed eaten, chalcid exit hole	Seed eaten, chalcid exit hole
36	Side	Empty seed	Filled seed
43	Side	Live larva	Live larva
44	End	Live larva	Seed eaten, chalcid exit hole
45	Side	Filled seed	Empty seed
46	Side	Live larva	Live larva

[1] Side = one seed positioned directly above the other.
[2] Angled = seed is angled slightly, so it is not squarely in its side or end.
[3] End = both seeds are visible; the flat surfaces of each seed form a 'line' between the two seeds.

Other limitations encountered in using X-ray imaging to determine levels of chalcid infestation in coriander seeds were: a) the chalcid exit hole was not visible in the image; b) the stage of insect development could not be determined; c) the viability of the insect could not be determined; and d) it was easy to mistake an underdeveloped seed for a chalcid-infested seed. Figure 2 is part of an X-ray image of Ames 23623. The notes for figure 2 reflect the actual contents of seeds based on hand dissection and were not determined by directly inspecting the image. When subsequent dissections were made, determination of seed quality and chalcid infestation based on the X-ray images revealed the limitations mentioned. However, many of these limitations may not exist for other seed and fruit types.

Coriander seed stored over LN_2 for 16 hours proved to be an effective method of killing chalcid larvae. This treatment did not adversely affect germination level, even for samples of low initial quality. Hand dissections and visual inspections verified the effectiveness of LN_2 in killing chalcids in coriander seeds. The LN_2 was effective in killing chalcid insects, regardless of the life-stage of the insect.

The use of X-ray imaging was shown to be useful for estimating insect infestations. However, the double-seed arrangement of coriander fruit made precise interpretations of X-ray images difficult. Single-seeded fruit types of other plant genera may be able to utilize this technology with greater success.

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