Production Steps to Reduce Seed Contamination by Pathogens of Cucurbits

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Summary. Selecting production areas for low disease pressure, implementation of preventive spray programs, and continuous monitoring for disease symptoms are important steps to keep seed production fields free of potentially seedborne diseases, such as bacterial fruit blotch of cucurbits (Cucurbitaceae), caused by *Acidovorax avenae* ssp. *citrulli*. However, seeds of cucurbit crops and other fleshy vegetables typically remain remarkably free of pathogenic bacteria and fungi while in intact fruit. The most significant risk for seed contamination comes at harvest when the inoculum present in the field or in the seed harvesting area may contaminate the seeds. Properly executed fermentation and seed drying processes significantly reduce seed contamination. Application of a no-rinse disinfectant formulation to freshly harvested seed just before drying may be the single most efficacious procedure to reduce the seed contamination risk. However, the disinfection step should not be expected to be effective unless applied as part of a fully controlled seed harvest process.

Cucurbitaceae is a large, taxonomically well-defined and isolated family, which includes several agricultural crops. The best-known representatives of the cucurbit family in the United States are watermelon (*Citrullus lanatus*) muskmelon (*Cucumis melo*), squash (*Cucurbita* spp.) and cucumber (*Cucumis sativus*).
Seeds of cultivated cucurbits are formed within fleshy fruit, which are typically enveloped by a harder layer, commonly referred to as a rind. The seeds are usually flat but vary in color and size among the cultivated species. Well-developed seedcoat surrounds two cotyledons and an oily embryo, the endosperm is typically consumed during seed development (Robinson and Decker-Walters, 1996).

Mainly due to the exposure to abundant organic substrates at the time of the seed harvest, commercial seeds of cucurbits typically harbor a large number of bacteria and fungi. Most microorganisms associated with cucurbit seeds are nonpathogenic and typically do not interfere with seed development.

While this article describes important steps to reduce the presence of potentially seedborne pathogens in seed production fields, information contained herein constitutes suggestions only and does not guarantee a disease-free crop. Despite following the best known production practices and seed testing for the presence of disease-causing pathogens, seed companies typically cannot and do not warranty seeds as disease free.

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Some microorganisms, such as those known as molds (*Aspergillus* spp., *Penicillium* spp.) and blights (e.g., *Pythium* spp., *Phytophthora* spp.), can invade seed/seedling tissues and thus become detrimental to seedling establishment only under specific environmental circumstances. Other microorganisms, such as the fungus *Didymella bryoniae* and bacterium *Acidovorax avenae* ssp. *citrulli* (*Aac*) have the ability to use healthy seed tissues as a substrate during seed germination and seedling development, and also have a potential to continue to parasitize adult plant tissues all the way to maturity, causing diseases known as gummy stem blight and bacterial fruit blotch, respectively.

Even though the onset of seed transmissible disease will largely depend on the conditions during seed germination and seedling development (Latin and Hopkins, 1995), the introduction of pathogens such as those causing BFB must be minimized to the maximum degree possible. On a large scale, seed company efforts are comprised of steps during seed production, seed conditioning/treating, and seed health testing. For the purpose of this review, we will mainly focus on the BFB pathogen and its watermelon host, bearing in mind that similar strategies can be useful in reducing the risk from several other seed-transmissible agents of cucurbit diseases.

Bacterial fruit blotch (BFB) of watermelon has been a menace to all segments of cucurbit industry since the first large outbreaks in the United States were associated with its seed-transmissible nature in 1989 (Rane and Latin, 1992; Latin and Hopkins, 1995). Even though the pathogen does appear to occur naturally in the United States (Walcott et al., 2000), a majority of reported epidemics in the United States originated from contaminated seeds. The bacteria causing BFB thrive in warm and humid greenhouse environment and can spread from a single seedling to the entire greenhouse in only a few days. This rapid spread is aided by overhead irrigation practices and is frequently promoted by a general lack of phytosanitary protocols in the greenhouse (Jarvis, 1992). If the contaminated transplants are taken into the field, the disease will typically follow its course and result in loss of yield and/or marketing value of the fruit at the end of the season. The losses can be greatly reduced by following appropriate phytosanitary recommendation but some loss may be inevitable under particularly favorable environmental conditions.

While developing effective disease risk-reducing strategies, the entire production process needs to be analyzed and the risk reducing measures implemented at each step. A relatively short growing cycle of cultivated cucurbits is commonly separated into five steps: 1) seeding or transplanting, 2) pre-flowering, 3) flowering (pollination), 4) postpollination (fruit/seed development), and 5) seed harvest. For the purposes of this review, we will additionally separate this seed production process into ten steps thought to be critical to securing disease-free seeds.

Planning and production phase

Pathogen-free stock seeds. Even considering the fact that majority of researchers (Latin and Hopkins 1995, Rane and Latin, 1992) and practitioners would agree that BFB is not a systemic pathogen, contaminated seeds can still serve as the vehicle by which the causal agent is introduced into seed production areas. The risk from developing BFB in production areas which are typically characterized by hot and dry climate is minimal, but there is a possibility that the pathogen may survive epiphytically without causing any disease symptom yet still contribute to seed contamination resulting in seed-transmissible disease. To avoid these scenarios, stock seeds should be produced in the areas associated with the lowest disease risk, and the seeds subjected to greater level of scrutiny during seed health testing compared to commercial hybrid seeds. To further reduce the risk from seedborne pathogens, it is also recommended that stock seeds be subjected to aggressive preventive decontamination treatments.

Selection of production area. Seed companies typically seek production areas with hot and dry climate during the entire growing season. Such regions are relatively easier to locate for cucurbits compared to other crops because of their relatively short (90 - 120 d) production cycle. This also allows flexibility in selecting specific parts of the year with optimal conditions in climates where year-round warm conditions allow multiple growing cycles.

Areas where there was no previous cucurbit seed production are considered lower risk because it is believed that the pathogen did not get a chance to become established. However, the ability to predict the risk from BFB is very limited because every production area will have its own unknown and unpredictable characteristics and one cannot assume that a "virgin" production area will carry a lower BFB risk compared to established production areas. It is important to consider the fact that the origin, epidemiology, and seed association of pathogens such as those causing BFB are not fully understood. The best long-term approach to reducing risk from their seed transmissibility is to focus on enforcement and follow up of strict production protocols and phytosanitary guidelines.

Phytosanitary measures during crop production. During production planning, one has to take into account all possible ways by which seed-transmissible pathogens may be introduced into the field, distributed within the field, make contact with seeds. Standard measures are to 1) favor drip over furrow irrigation, 2) apply fungicides and bactericides on a fixed (e.g., every 2 weeks) schedule, and immediately following rainfall, and 3) carefully monitor and control insect populations. More specific recommendations are as follows:

Transplant production. Transplanting is a preferred method for seed crops because a more uniform crop and more efficient use of valuable stock seeds can be achieved through using transplants. Transplants are typically raised in greenhouses, which even in dry areas, can have high levels of relative humidity making it necessary to apply all precautions necessary to reduce the onset and transmission of plant diseases (Latin et al., 1995). The facility where seedlings for seed production will be raised should be dedicated to stock seeds and not planted to any other seeds at the same time. The seedlings should be inspected at least twice by personnel trained in disease recognition, and preventively treated with bactericides just before transplanting. If BFB or any other potentially seed-transmissible disease is confirmed on transplants, it is recommended that the entire operation be suspended.

Prepollination. During the first

part of the cycle, in most production areas, conditions are relatively cooler and wetter and therefore more conducive for disease development compared to the latter part of the cycle. A minimum of one field inspection is recommended for each melon seed crop before pollination along with the regular application of bactericides. Even a minimal amount of disease that occurs at this stage is likely to be effectively transmitted to the entire crop through manual pollination of the crop during pollination. This is a much lesser concern for open-field seed production protocols.

Pollination. These operations are very high risk for spreading bacterial and other diseases that can be dispersed through manual contact. A helpful strategy to limit the scale of the problem is to assign pollination workers specific field areas (e.g., a certain number of rows) to limit disease spread. Recent reports (R. Walcott, personal communication) indicate that several different bacterial species, including *Aac*, can invade internal watermelon fruit tissues and seeds following inoculation of stigmas at the time of flowering. The possibility was raised that bees could serve not only as pollination agents but also as disease vectors. Considering that insect pollination is practically not used in Asian seed production areas, which have historically originated most BFB-contaminated commercial seeds, the practical significance of this mode of seed invasion is doubtful. However, there is still a possibility that bacteria could be introduced through manual contact by the workers performing hand pollinations. Even though there is no empirical evidence that this way of seed colonization is responsible for seed transmissible BFB (B. Lovic, personal observations), ongoing research investigating this mechanism merits attention from seed producers and fruit growers alike.

Postpollination. Management practices are limited to maintaining disease- and insect-free crops, with minimal interference with crop growth and development. Preventive application of bactericides following each rain is helpful but at later stages of crop development, plant tissues become increasingly inaccessible to aerially applied pesticides.

Documenting phytosanitary measures is an important part of a long-term strategy to reduce seedborne disease risk. By keeping a log of all conditions associated with the risk (e.g., irrigation schedule, rain frequency and duration, and relative humidity), seed producers provide valuable insight to seed companies that their recommendations are fully implemented.

FIELD INSPECTIONS. Field inspections, regardless how thorough they are, do not guarantee pathogen-free seeds. However, the inspections are useful in documenting absence of disease symptoms from the field as well as compliance with other recommended risk-reducing measures. To get most value from field inspections, they should be conducted by personnel competent in recognizing disease symptoms. Samples should be taken and analyzed, and all steps of the process well documented. It is also recommended that multiple inspections be conducted because the pathogen can often be detected only in certain parts of the growing season without necessarily leaving any signs that could be visible at the end of the cycle. Some seed certifying agencies (e.g., California Crop Improvement Association, Davis, Calif.) offer field inspection and staff training services.

Seed harvesting phase

While the circumstances during the seed harvest process may not be the only way in which bacteria gain contact with the seeds, empirical evidence strongly suggests that the risk from seed-transmissible BFB can be either minimized or favored through various steps of the seed harvest process. Empirical evidence also suggests that the areas associated with the greatest risk are not those characterized by disease-conducive conditions or inadequate field phytosanitary practices but those that are characterized by similar seed harvesting methodology. Seed harvest of cucurbit crops provides ample opportunities for spread, growth, and reproduction of bacteria and fungi. Seeds of cucurbits are borne within fleshy fruit and the process of seed harvest calls for breaking the surface of the rind and separation of seeds from the flesh before seeds are washed and dried. Each step in this process carries a certain risk that the seeds either be contaminated with bacteria causing BFB or that the numbers of seedborne bacteria increases to seed-transmissible levels.

Seed harvesting requires either highly specialized and expensive equipment (e.g., mechanized seed harvesters, forced/heated-air dryers) or large number of skilled and reliable labor. Seed production areas that originated the greatest number of known cases of BFB-contaminated seeds have unquestionably been those where seeds are harvested manually and sun-dried rather than those areas where seed harvesting, washing, and drying equipment was available to make these processes more reliable.

FRUIT SELECTION AND TREATMENTS. Seeds of fruity vegetables such as cucurbits are typically free of bacteria while in intact fruit (Neergard 1977). Though there is evidence that bacteria, including pathogenic strains of *Pseudomonas syringae* causing angular leaf spot disease of cucurbits (Kritzman and Zutra, 1983), can be found on/in seeds isolated from intact cucurbit fruit (Mundt, 1976), most researchers agree that *Aac* and similar bacteria do not invade fruit systemically (Giles-Frankle et al., 1993; Latin et al., 1995; Latin and Hopkins, 1995). Association of the pathogen with the seeds is thought to be mediated either through extension of fruit surface lesions into the fruit, or when seeds are mixed with the inoculum present on rinds, leaves, other plant tissues, and/or other potential still undetermined sources in the process of seed extraction.

To minimize the possibility that fruit or other plant material serve as a source of inoculum leading to seed contamination, the optimal seed harvesting process should include 1) visual inspection to select only the unblemished fruit for seed harvest, 2) collection and transport of fruit from the field, and 3) decontamination of the fruit surfaces prior to seed harvest. Ability of the decontamination agent to partially penetrate rind tissues would be particularly desirable for this application. Heat has such a characteristic, and flaming the surface of the fruit using propane burners has been described (Lovic, 1998) and evaluated for this purpose. This method was found difficult to implement in practice due to very large quantities of fruit that are processed at the time of the seed harvest (B. Lovic, personal observations). Liquid surface disinfectants would be expected to be less efficacious but could be used for this purpose.

FERMENTATION. Fermentation is a process of microbe-mediated bio-

chemical degradation of juices and pulp associated with the freshly harvested seeds. Following extraction from the fruit, seeds are incubated in the presence of residual juices and pulp, typically for a period of 24 h before they are washed. Fermentation not only helps with breaking down the organic matter and thus facilitates seed washing process; it also reduces the risk from seed-transmissible diseases such as BFB (Hopkins et al., 1996). Fermentation should therefore be employed whenever possible. Unfortunately, fermentation can have a negative impact on the quality of certain seed types such as triploid watermelon seeds.

SEED WASHING AND DISINFECTION. Cucurbit seeds require washing in large volumes of water to remove the organic matter before they are dried. In seed producing regions of California and other developed agricultural regions, specialized vine seed washing facilities are available which allow large batches of freshly harvested or fermented seeds to be washed quickly. In less developed, remote agricultural areas like those typically used for seed production in Asia, small batches of seeds are washed by individual growers. The latter scenario creates a risk that the seed transmissible bacteria associated with the seeds will remain, or that the seeds could be contaminated during this process. To reduce this risk during manual seed washing, it is recommended that 1) only clean well water be used for seed washing, 2) the seed washing process be conducted as quickly as possible, and 3) the seeds be disinfected during the last stage of the washing process and then be dried immediately.

Aac is not particularly resistant to disinfectants and a number of chemical disinfectants can effectively eliminate it from water solutions. However, the challenge of removing them from the seeds is much more formidable. The best-known and most readily available chemicals previously evaluated for this purpose are sodium hypochlorite and hydrochloric acid. Hydrochloric acid has been evaluated for purposes of seed disinfection and it was determined that a 15-min treatment with 1% hydrochloric acid can effectively reduce seed transmissible *Aac* from contaminated seeds in a repeated experiment (Hopkins et al., 1996). However, all cucurbit seeds need to be rinsed after hydrochloric acid treatment and certain cucurbit seed types (e.g., triploid watermelon seeds) can be damaged by this treatment even if they are rinsed.

Recently, peracetic acid (Baldry, 1983; Mari et al., 1999) based products (e.g., Tsunami 100; Ecolab, Mendota Heights, Minn.) were evaluated as seed disinfectant and determined to be appropriate for disinfection of freshly harvested cucurbit seeds (Hopkins et al., 2001). Peracetic acid products were lethal to *Aac* in water suspension after a 30-s exposure at 80 $\mu g \cdot mL^{-1}$ (ppm), yet did not show reduction in seed quality to sensitive seed types even after soaking the seeds for 30-min at 1600 $\mu g \cdot mL^{-1}$. The peracetic acid-treated seeds do not need to be rinsed after treatment, which simplifies the field protocols and extends the disinfection effect into the drying process. Further, liquid that remains after the standard treatment has a pH above 2.8 and the active ingredients rapidly react with soil and decompose to water, acetic acid, and oxygen, so it can be safely disposed of by spreading over soil surfaces.

The undesirable characteristic of peracetic-acid based products is that they are highly corrosive and reactive, and thus are subject to a variety of restrictions during transport and storage. Working solutions are practically nontoxic but manipulation of concentrated solutions requires worker safety training and use of protective equipment (e.g., goggles, gloves, apron).

SEED DRYING. Seed drying is critical to many aspects of seed quality, including the risk from seed-transmissible bacteria and fungi. If pathogenic microorganisms such as *Aac* are present on the seeds at the time seed drying is initiated, it is likely that their populations will increase as long as seed moisture content will support their growth and reproduction. Therefore, it is important that, especially initially, the seed drying process be as efficient and rapid as possible without impacting other aspects of seed quality.

There are two main ways in which cucurbit seeds are dried following extraction and washing: forced air drying and sun-drying. Two typical devices used for forced-air seed drying are drying tables and rotary seed dryers. In each device, heated air, typically generated by a propane burner, is forced to allow fast and uniform drying almost entirely independent of area environmental parameters. During sun-drying, which is typically practiced in most third-world production areas, the rate of seed drying depends on local weather parameters (solar radiation, relative humidity, wind). The seed drying time typically can vary from about 6 h to 3 d, depending on the exact combination of key parameters and seed characteristics. To reduce the risk from negative impact of prolonged seed drying, historical climate information needs to be documented specifically for the seed harvest period prior to placing the production in a new area. Also, the seed harvest should not be initiated at the beginning of periods of overcast, cold weather. Other helpful measures for increasing the efficiency of seed drying protocols are: 1) centrifuging the seeds before drying to eliminate surface moisture, 2) drying the seeds in thin layers, 3) frequently mixing the seeds, and 4) elevating seed drying screens and placing them in optimal locations. Still, sun-drying will always be at a disadvantage compared to forced-air drying protocols and the goal should be to secure availability of forced-air drying equipment at all cucurbit seed production locations.

Postharvest phase

SEED TRANSPORT, MANIPULATION, AND STORAGE. It is difficult to envision a scenario where pathogenic bacteria would contaminate the seeds during processing of dry seeds but it is recommended that the seed processing equipment be periodically cleaned and disinfected. It is also important not to allow contamination by saprophytic fungi (e.g., *Rhizopus* spp., *Penicillium* spp.), which could potentially interfere with seed health testing protocols or seed germination.

SEED SAMPLING. An appropriate sampling strategy needs to be defined at the time of crop planning and has to take into account all factors which will potentially impact the composition of microbial populations associated with dry seeds. For some diseases, such as those caused by insect-transmissible plant viruses (e.g., squash mosaic virus) the sampling strategy will be rather simple since most operations in the field (e.g., pollination timing, seed harvest parameters) will not affect the likelihood of seed transmissibility. However, for pathogens such as *Aac,* whose chances for seed transmissible occurrence, as outlined above, are pro-

foundly affected by the timing and protocols of individual operations, the sampling strategy can and should be complex.

A general rule is that the number of lots to be sampled should be directly related to the capacity to perform individual operations on a large scale. For example, if a single hybrid is planted into a 5-ha (12.4-acre) field on the same day, and all subsequent operations (irrigation, fertilization, pollination, seed harvest, washing and drying) are conducted simultaneously on the entire crop, there is no reason not to consider all the seeds produced in that field as a single lot and obtain a single sample. However, in production areas in Asia (assuming manual production and harvest protocols), individual fields are either very small or large fields are divided into small units which are each handled by an individual grower, or typically, grower family. One grower can handle only up to 0.3 ha (0.75 acre) at the time of pollination. Since each grower will conduct individual operations on different dates and using different protocols, it becomes necessary that the seeds produced by individual growers be considered separate lots, and therefore sampled and tested separately. One could even make a justification that, if a single grower harvests her/his crop over a period of 10 d, each day's harvest should be considered a separate lot. So, in order to subject the seeds harvested from 5 ha in Asia to the same scrutiny as the seeds produced in the same-size field in California (assuming fully mechanized production and harvest protocols), it becomes necessary to take many more samples.

Lot definition and seed sampling strategy are at the heart of the apparent association of seed transmissible BFB with the seeds produced in China and some other Asian countries. Seed production is typically placed in the areas where diseases such as BFB are practically not known to occur, rigorous phytosanitary regimes are enforced, and the fields regularly inspected and judged to be free of any symptoms. However, the sampling strategy rarely adequately reflects the variability of conditions under which individual seed crops are produced and harvested as related to the risk from seed-transmissible BFB. Adequate sampling strategy is difficult, perhaps even impossible, to achieve because, eventually, the increased cost of sampling and seed health testing becomes prohibitive. The only way to solve this problem is through harmonizing production and seed harvest protocols towards increasing the sizes of individual lots. This will not only reduce the number of samples and seed health tests but will also enable better control of the seed production process.

Seed health testing is beyond the intended scope of this review, however, electing an adequate seed testing method and its appropriate execution is fundamental to seed production. Seed testing is the only way to assess the impact that changes in seed production practices have on seed-transmissible disease incidence. A number of seed testing methods for seedborne *Aac* have been researched and proposed in the past (Walcott et al., 2000). However, the method which remains in use and is still recommended by American Seed Trade Association (Washington, D.C.) and International Seed Testing Association (Zurich, Switzerland) is a so called growout method where a specific number of seeds are planted and grown for three weeks under conditions conducive for disease development. This method is difficult to control, takes a month to complete, and is very expensive, but the reliability of any alternative methods has not been established through adequate comparative testing process. The challenge of any alternative direct seed assays lies in the fact that plant exudates induce radical qualitative and quantitative shifts in microbial populations associated with dry and germinating seeds (Cottyn et al., 2001).

Finally, it would be highly desirable if seed health would become part of the seed certification process offered by independent agencies. The California Crop Improvement Association (Davis. Calif.) has made significant progress over the last years toward enhancing the value of their services by offering field inspections and training of seed production company personnel in disease recognition and diagnosis. Extension of these efforts into auditing other important steps in the seed production process will further enhance the value of such services.

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