Managing major greenhouse vegetable diseases using biological and molecular techniques

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Summary: Greenhouse vegetable crops are grown worldwide as a source of nutrients and fibre in the human diet. Fungal plant pathogens can cause devastating diseases in these crops under the appropriate environmental conditions. Greenhouse vegetable growers confronted with the challenges of managing fungal pathogens have the opportunity to utilize microorganisms as biological control agents. A number of commercially available biological products have demonstrated significant disease reduction. The use of molecular techniques in biotechnology to identify and diagnose plant pathogens is becoming increasingly valuable.

Introduction: Vegetable crops (tomato, cucumber, pepper and lettuce) are produced as both fresh market and processed commodities and are grown in controlled environments, such as glasshouses or other similar structures.

There are numerous fungal diseases that attack a wide range of these vegetable crops, thereby reducing crop yield and quality. Methods for disease control have included the use of cultural practices to reduce pathogen inoculum and disease incidence, development of resistant cultivars, and the application of chemical fungicides to inhibit pathogen development. The use of biological control strategies has also demonstrated the potential of fungi, bacteria and viruses in reducing a range of fungal pathogens that cause various diseases on vegetable crops. Rapid and early diagnosis of diseases would help control the spread of devastating diseases in greenhouses. Identification of pathogens from culture, tissue, soil, or air samples would also help in understanding its epidemiology in greenhouse crops. Conventional identification of pathogens from commercial greenhouse samples, using morphological parameters and pathogenicity tests, needs culturing, purification and subsequent knowledge of taxonomy and it is time-consuming.

Use of molecular techniques has facilitated identification of different microorganisms quickly, efficiently and reliably and thus has helped in disease management decisions.

In this paper, some examples of recent successes in biological control of fungal diseases of greenhouse vegetable crops using fungi, yeasts, and bacteria will be discussed. In particular, the research done at the Pacific Agri-Food Research Centre of Agriculture and Agri-Food Canada in Agassiz, British Columbia, will be presented. In addition, the utilization of molecular techniques for quick detection and identification of fungal pathogens for disease control will be reviewed.

The diseases to be considered here for which biocontrol strategies have been developed include those caused by pathogens that infect the roots/crown of greenhouse vegetable crops. These include fungi such as Pythium sp., Rhizoctonia solani and Fusarium oxysporum. A group of foliar infecting fungi of vegetable crops, which cause leaf spots and blights and stem infection, also have biological control strategies developed against them. These include Botrytis cinerea (grey mould), Didymella bryoniae (gummy stem blight), and a new disease called internal fruit rot on pepper caused by Fusarium oxysporum f. sp. capsici.

Many different fungal, yeast and bacterial biological control agents have been identified and evaluated for disease control potential against the above-mentioned pathogens, and some have been formulated and brought to market to provide disease control options for producers of vegetable crops. The use of biological control agents may be particularly attractive for vegetable crops grown in glasshouses due to the high market value of these crops and the possibility for control of environmental parameters, particularly temperature and relative humidity. These are important variables that can significantly influence the efficiency of biological control agents under greenhouse conditions.

The rationale for development of

Greenhouse CAR403A, February 2005 — 7
biological control agents against fungal diseases on vegetable crops was to provide an additional/alternative approach to augment/replace the use of chemical fungicides, to provide a level of disease control in the absence of crop genetic resistance, and to supplement cultural control practices to further minimize the impact of these diseases and reduce chemical residues in food.

BOTRYTIS STEM CANKER

Botrytis cinerea, causal agent of stem canker on tomatoes, is renowned for its broad host range; over 200 species can be infected, resulting in considerable economic losses. On greenhouse tomatoes, this fungus causes serious and widespread stem canker and grey mould diseases. This pathogen infects leaves, peduncles, stems and fruits of the tomato crop. Infection of leaves and stems is characterized by large, irregular, brown lesions sometimes with masses of grey-brown spores.

This fungus can exploit fallen tomato petals as food sources from which it can invade green or ripening tomato fruit, either directly or by first attacking calyces attached to the fruit. Under favourable conditions in a commercial greenhouse, sporulation on lesions can be considerable with cycles of sporulation generating a fast developing epidemium within the crop.

Molecular technique: A dot blot assay, based on the internal transcribed spacer of the ribosomal region, was developed for rapid diagnosis and identification of Botrytis cinerea, causal agent of grey mould of greenhouse tomatoes (Mathur and Utkhede 2002).

In this assay, DNA from fungal cultures and diseased plant tissue was amplified by PCR using primers specific to septate fungi, fixed to nylon membranes, and hybridized with digoxigenin-d-UTP labelled oligonucleotides specific to B. cinerea. The hybridized probes were detected by chemiluminescence.

Of four probes screened for reaction, the probe B01 gave a positive reaction with all isolates of B. cinerea and with fresh or frozen grey mould diseased plant tissue from Research Centre greenhouse and commercial greenhouses. All other greenhouse fungi tested negative with this probe B01. This dot blot diagnoses infection by B. cinerea in large numbers of samples rapidly and reliably and distinguishes isolates of B. cinerea from other fungi found on greenhouse crops.

Biological control: Experiments were conducted to identify potential antibiotic-producing biological control agents by in vitro dual culture tests and to evaluate selected biological agents for control of stem canker caused by Botrytis cinerea on tomato plants grown in yellow cedar sawdust in a research greenhouse (Utkhede et al. 2001).

Lesions in curative treatments with RootShield and a strain S33 of yeast (Rhodotorula dioica) were significantly shorter compared with the inoculated control. Plants treated with RootShield or S33 had significantly higher total fruit yield than the inoculated control. The treatments RootShield, SoilGard, and S33 produced significantly more fruit than the inoculated control. The number of dead plants was significantly lower in treatments with RootShield and S33 compared with the other treatments and the inoculated control. In another set of experiments, Precise® and S33 applied as sprays prevented the occurrence of stem canker and increased the fruit yield of tomatoes (Utkhede and Mathur 2004). The number of dead plants was also lower with these treatments compared with the other treatments and the inoculated control.

Additional experiments have shown that hen egg white lysozyme (HEWL)
533 and Azauxystrobin significantly reduced lesion lengths caused by B. cinerea on cucumber plants (Utkhed he and Boughnoff 2003). HEWL, plus 533 applied together did not have any effect on this cucumber disease. Nov 4 controlled Botrytis stem canker but not gummy stem blight.

**Didymella Gummy Stem Blight**

Gummy stem blight of cucumber is caused by the fungus Didymella byssothor ae. The first symptom on greenhouse cucumber is lesions on stubs left after the removal of fruit, tendrils or lateral shoots. These lesions may elongate and girdle the stem, causing wilt and eventual death of the plant. It is one of the serious diseases of greenhouse-grown cucumbers in the Netherlands, where it causes fruit rot.

Gummy stem blight is also a problem in other European countries, including the U.K. and Denmark. A small to moderate degree of resistance does exist in cucumber germplasm, but no gene for resistance has yet been identified. Adequate control is hard to achieve with present fungicides because plants grow fast, have dense foliage, and are continuously being wounded by picking and trimming.

This disease has become a serious problem in commercial greenhouses in Canada. Pesticides ‘Rova’, ‘Boslon’, ‘Dymine’ are registered for control of this disease in Canada. However, these pesticides are not very effective in controlling this disease. This may be due to the development of resistant pathogens.

**Molecular Technique**: We have developed a dot blot technique (as described earlier) for rapid diagnosis and identification of Didymella byssothor ae, causal agent of gummy stem blight of greenhouse cucumbers (Koch and Utkhehe 2002).

After preliminary testing, probe D6 was selected for further study. Probe D6 gave a positive reaction with all isolates of D. byssothor ae and Plasmod ios cactorum tested and with fresh or frozen gummy stem blight diseased plant tissue. All other greenhouse pathogens tested gave a negative result.

Cucumber stem tissue infected with Botr nis cinerea and healthy cucumber stem tissue also gave a negative result.

**Use of molecular techniques has facilitated identification of different micro-organisms quickly, efficiently and reliably, and thus has helped in disease management decisions.**

**Biological Control**: Five experiments were conducted to test chemical and biological treatments to control gummy stem blight of cucumber caused by Didymella byssothor ae (Utkhed he and Koch 2002). The chemical treatments ‘Nov 4’ and Azauxystrobin controlled the disease in three experiments and kresoxin-methyl
in greenhouses, is an important crop in Canada and around the world. About 40% of orange pepper fruits of the cultivar "Symphony" were observed to be infected with Fusarium fruit rot in a commercial B.C. greenhouse during 2001, and 1% in 2002 (Utkhede and Mathur 2003).

In 2003, the disease was observed on the cultivars "444" and "Spirit" in a few commercial greenhouses in B.C. and Alberta. The disease appeared on mature fruits at harvest time and affected fruits were considered as culls. Infected fruits were unmarketable due to external symptoms and internal rot, resulting in losses in commercial growers.

The disease appeared as discoloured soft patches or necrotic spots mostly at the calyx end and sometimes anywhere on the mature fruit at harvest time. Seeds and surrounding area inside the fruits were covered with fungal growth and orange pink spore masses.

Fungal isolations were made from the lesions. The fungus was identified as Fusarium subglutinans by Dr. Keith Setoert of the Eastern Cereal and Oilseed Research Center in Ottawa. To confirm pathogenicity, flowers and developing fruits of sweet pepper cv. "Symphony" were inoculated with F. subglutinans. About 85% of inoculated fruits and flowers developed symptoms on fruits similar to naturally infected fruits at maturity. Fruits from control plants did not develop any disease. On agar media, F. subglutinans was recovered from all inoculated infected fruits.

Preliminary studies showed that this pathogen does not infect other greenhouse crops like tomatoes, cucumbers or brassica. To our knowledge, this is the first record of fruit rot caused by F. subglutinans on greenhouse sweet peppers (Utkhede and Mathur 2003).

Additional experiments were conducted to elucidate the mode of pathogen transmission and to determine the effect of the inoculum's concentration, growth stage, and various pepper cultivars on disease development (Utkhede and Mathur 2004).

**Symptoms of Fusarium subglutinans internal fruit rot on peppers.**

Inoculum concentrations of 10^4 to 10^6 conidia/ml, resulted in a higher incidence of fruit infections compared to 10^0 when just-opened and fully-opened flowers were inoculated. Flowers inoculated with F. subglutinans at different stages developed more disease than compared to fruit inoculations. None of the seeds from infected pepper fruits germinated and 92% yielded typical F. subglutinans colonies. No evidence of root infection of pepper plants by this pathogen was observed. Fruits of 'Bosco' and 'Mardusa' were less susceptible to this disease than '444' and 'Symphony'.

**Molecular Technique: Rapid and accurate detection of F. subglutinans using rDNA blot hybridization was developed.** Mathur and Utkhede (2004). Out of six probes tested, two probes (Fsh-3 and Fsh-5) were selected because they differentiated F. subglutinans from other Fusarium species and greenhouse pathogens. These probes can discriminate F. subglutinans from other Fusarium sp. as well as other fungal pathogens causing fruit rot of peppers. This technique would help to detect and identify accurately F. subglutinans in culture and pepper fruits.

**Biological Control: An experiment was conducted in 2003 to evaluate biological and chemical pre-harvest treatments for control of internal fruit rot of peppers caused by Fusarium subglutinans. The inoculum of F. subglutinans was deep inoculated on flowers of sweet peppers cv.
Cucumber root rot on cucumber.

Cucumber root rot is a common disease of cucumbers caused by Pythium spp. The disease is characterized by the presence of dark brown to black, circular to oval lesions on the roots. These lesions can grow into larger areas of decay, eventually leading to the death of the plant. The disease can be managed through cultural practices such as crop rotation, good drainage, and the use of pathogen-free soil. Chemical controls include the use of fungicides, but care should be taken to avoid overuse due to the risk of resistance development.
pathogens of lettuce in recirculating hydroponics systems.

Once these pathogens are introduced in the system, their control is very difficult and sometimes the grower has to shut down the entire system. Tiny feeder roots of lettuce produced in hydroponics systems can be infected by *P. syringae* spp. *alcaligenes* and *P. syringae* sp. *solanacearum*. These pathogens affect the growth and yield of lettuce, and can result in plant death. At present, there are no control methods available for this disease. The use of fungicides in a greenhouse environment can interfere with insect biological control programs and at present, there are no effective fungicides registered for use on cucurbits grown in soilless culture.

**Fusarium root rot**

Fusarium root rot is caused by *Fusarium oxysporum f. sp. radicis-mericurii* (FORC), a new disease on greenhouse cucumbers that affects both yield and quality, and can result in plant death. At present, there are no control methods available for this disease. The use of fungicides in a greenhouse environment can interfere with insect biological control programs and at present, there are no effective fungicides registered for use on cucurbits grown in soilless culture.

**Biological control**

Three composts — Coop, Dairy, and Vermi made from dairy and animal manures — were tested for suppression of root and crown rot, and for the enhancement of seedling growth and fruit yield of cucumber (Kamangara and Ukbede 2000). Several bacterial isolates from the composts were antagonistic to the root rot pathogen and significantly increased the dry weight of four-week-old seedlings. The effect of compost on fruit yield was determined using compost-amended sandsoil, seaweed, and without inoculation of root rot pathogens. In un inoculated plants, the composts Coop, Dairy, and Vermi increased the cucumber fruit yield by 12, 13, and 12%, respectively. In the inoculated plants, yield was reduced by 23% compared to the uninoculated sandsoil. The biggest increase (37%) in fruit yield was observed, irrespective of FORC inoculation, when the Dairy compost amended sandsoil was inoculated with a biological agent *Bacillus subtilis* strain BACTO.

**Conclusions**

The potential use of microbial biological control agents for control of fungal diseases on greenhouse vegetable crops is reviewed. These microbial agents are easy to grow and can be applied as a seed treatment, an addition to nutrient solutions, or as a foliar spray. Some of the microbial agents appear to protect plants against a wide range of pathogens and the potential for commercial utilization is promising.

The biological control agents are generally most effective when applied as a preventative treatment, prior to or at the onset of disease, and multiple applications may be needed to provide long-term disease suppression. At high levels of disease pressure, biological control agents can be anticipated to perform less well.

Some of the agents may be used in combination with, or in association with, chemical fungicides, if it can be demonstrated that their survival is not adversely affected. Similarly, it may be possible that combinations of biological and chemical agents may be more effective than single agents alone as little research has been done in this area. Biological control agents that affect more than one disease should have greater market potential than those that specifically target a particular disease. One such example is that of *P. syringae* which has the potential to control Botrytis stem rot, *Didymella* gummy stem blight, *Pythium* root rot, and *Fusarium* fruit rot. The use of microbial biological control agents has generated significant interest in scientific research and product development to ensure that commercially viable products will continue to be brought to market.

Correct identification of fungi by conventional means typically

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(Ananyo) significantly increased fresh and dry lettuce weight compared with the control. These treatments also significantly reduced the disease ratings compared with the control.
requires several days. Molecular methods of identification have been developed which reduce the time required for a 4-day result to a day or two. Molecular methods using the dot blot technique would allow rapid diagnosis of disease in infected tissue from growers' greenhouses, and would facilitate breeding resistance against plant diseases. Rapid, accurate disease classification allows for timely disease control, and for rapid assay of propagating materials when developing disease-resistant plants. In addition, the dot blot assay could be valuable for epidemiological and etiological studies, e.g., to monitor mycelial growth through the infected plant.

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