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REVIEW ARTICLE

Status, Prospects and Challenges of Yeast as Bio-Control Agent in Management of Post-Harvest Diseases of Fruit Crops

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ABSTRACT

Post harvest losses of fruit crops in India are high due to varying temperature and humidity conditions. Losses in fruits are estimated to vary between 20 and 30%, valued at nearly 8000 crores annually, depending on the variety of fruit and the postharvest handling system. The application of fungicides to fruits after harvest to reduce decay has been increasingly reduced due to the development of resistance in phytopathogens to many key fungicides, lack of new molecules, negative public perception regarding the safety of fungicides and consequent restrictions on fungicide use. Biological control of postharvest diseases has emerged as an effective substitute and several biocides are available in the market. One of the foremost constraints with biological management of postharvest disease is inconsistency in the efficacy of the formulations. The limitations of biocontrol products can be addressed by enhancing biocontrol through genetic and environmental manipulations and integration with other alternative methods that, alone, do not provide adequate protection but, in combination with biocontrol, provide additive or synergistic effects.

Keywords *Post harvest losses, resistance, Biological control, biocides and Yeast*

It is predicted that by 2050, the world's overall populace will reach approximately 9 billion people. Therefore, to feed this increasing world population, a raise of about 70% in agricultural food production is necessary (Raney, 2009). Problems like global warming, environmental pollution and climate change has lead to various kinds of biotic and abiotic stresses in plants which are responsible for significant yield loss to a large extent and it is an issue of major concern for the wellbeing of mankind. A study on 'Postharvest Food Losses in Developing Countries' conducted by a committee of the US National Research Council concludes that, 'postharvest losses are "enormous"'. The committee extrapolated from apparent loss patterns and expected production trends and projected postharvest food losses to be, at a minimum, 47,000,000 Mt of durable crops and 60,000,000 Mt of perishable crops. 'The average minimum losses reported for roots, tubers and fruits, vegetables were 16 per cent and 21 per cent, respectively; many more "qualitative" references, not included here, indicate estimates of 40–50 per cent and above.' Biotic stress factors involve fungi, bacteria, virus, nematodes weeds, and insects, which cause a yield loss up to 31–42% (Moustafa-Farag *et al*., 2020). Among them, fungal pathogens are the most severe limiting factor for crop production worldwide. Greater than 10,000 spp. of fungi are considered as responsible for a broad spectrum of plant diseases. Consequently, chemical fungicides are still employed injudiciously as a major means of disease management. These are not only expensive, but their application results in the accumulation of harmful toxins in soil and groundwater in our ecosystem (Raju *et al*., 2003 and Atreya *et al*., 2012). Moreover, the indiscriminate use of fungicides compels the pathogens to undergo genetic mutations which are eventually ascribed to the selection of fungicide resistant biotypes. For instance, *Venturia inequalis* (Meszka *et al*., 2008), *Phytophthora infestans* (Matson *et al*., 2015), *Colletotrichum musae* (Slabaugh *et al*., 1982) and *Colletotrichum gloeosporioides*, *Diplodia natalensis*, *Phomopsis citri* (Spalding, 1982; Farungsang and Farungsang, 1991) turn resistant to dodine, metalaxyl, benomyl and benzimidazole, respectively. Recently, stakeholders have shown keen interest towards the development of ecofriendly and cost-effective strategies for plant disease management (Panth *et al*., 2020). Biological control mechanisms are contemplated as significant measures for disease management because chemical fungicides adversely affect other non-target organisms (Köhl *et al*., 2019). There are several bodies of evidence which support the fact that some microorganisms cause growth inhibition of pathogenic spp. by impairing their metabolisms and/or establishing a parasitic relationship (Panth *et al*., 2020). Additionally, the application of

biological control agents (BCAs) with reduced concentrations of chemicals stimulates disease suppression in a similar manner to high doses of chemical fungicide treatments (Hyder *et al*., 2017). Cook and Baker (1983), in their book on biological control, cited only one example of the biocontrol of postharvest disease of strawberry fruit rot using *Trichoderma* sp. Subsequently, Wilson and Pusey (1985) presented their initial research on *Bacillus subtilis* to control brown rot on peaches, caused by *Monilinia fructicola*, and the organism was patented. A number of microorganisms (bacteria, yeasts and fungi), which effectively control postharvest pathogens, have been identified for the control of postharvest diseases and some of these have been patented and registered (El-Ghaouth and Wilson, 1997 and 2002). In several studies, yeast strains (*Aureobasidium pullulans*, *Candida oleophila*, *C. guilliermondii*, *C. sake*, *Cryptococcus laurentii*, *Debaryomyces hansenii*, *Metschnikowia pulcherrima*, *Pichia gulliermondii*, *Sporobolomyces roseus*) are reported for biocontrol of postharvest fungal decays of fruits caused by *Alternaria alternata*, *Botrytis cinerea*, *Geotrichum candidum*, *M. fructicola*, *Penicillium digitatum*, *P. italicum*, *P. expansum* and *Rhizopus stolonifer* (Droby *et al*., 1989; Wisniewski *et al*., 1991; Sharma, 1992, 1993, 2000; Mehrotra *et al*., 1996, 1998; Sharma *et al*., 1997; Spadaro *et al*., 2002) . In the past 25 years, research on biological control of postharvest diseases has moved from laboratory to practical applications (Wisniewski and Wilson, 1992; Wilson and Wisniewski, 1994; Mari and Guizzardi, 1998; Droby *et al*., 2001; Janisiewiez and Korsten, 2002; Korsten, 2006). By early 2000, there were three postharvest biological products available in the market: Aspire™, a product developed from *C. oleophila* (limited to the USA and Israel); BioSave™, developed from *P. syringae* to control decay caused by *P. italicum* and *P. digitatum* (limited to the USA); and Yield- Plus™ (limited to South Africa). Avogreen™, a commercial product of *B. subtilis*, was developed to control diseases caused by *Cercospora* spot and anthracnose of avocado.

Collection of Antagonistic Yeast

Often, phyllosphere *viz*. carpel, phylloplane of flowers and fruits, in a few cases, other matrixes have provided the major source for collection of antagonists (Filonow *et al*., 1996; Sharma, 2003; Belve *et al*., 2006). Various strategies have been employed to isolate antagonists and these include isolation from natural cracks on the fruit surface; agar plates containing apple juice that were seeded with a rot pathogen (Wilson *et al*., 1993); freshly made wounds on apples in the orchard that were exposed to colonization by fruit-associated microbiota from 1 to 4 weeks before harvest (Janisiewiez, 1996); and from an apple juice culture resulting from seeding diluted apple juice with the orchard-colonized wounds and repeated reinoculation to fresh apple juice. Isolation of the antagonists can be improved by using fruit from unmanaged orchards (Falconi and Mendgen, 1994) where natural populations have not been disturbed by chemical usage and the pool of potential antagonists is greater than in a chemically managed orchard (Smolka, 1992). Natural microbiota maintains a balance among the microbes normally present and inhibits the growth of newer invaders. Sharma (2005) reported that undiluted fruit washings when plated on agar plates exhibited a dense population of yeast and bacteria and, on dilution, filamentous fungi of the pathogenic type were isolated. This suggests that bacteria and yeast, naturally present on the surface, may inhibit the growth of other microorganisms, including plant pathogenic fungi. Later, it was observed that the citrus fruits, when washed and stored, rotted faster than the unwashed fruits, suggesting that these bacteria and yeast provide protection to fruits against postharvest pathogens. Rather than *in vitro* screening of organisms in Petri plates, which favoured the identification of antibiotic-producing organisms, a selection strategy was developed to identify suitable yeast antagonists (Wilson *et al*., 1993). The method involved placing washing fluids obtained from the surface of the fruit into fruit wounds that subsequently were inoculated with a rot pathogen. Organisms were then isolated from the surface of wounds that did not develop infections. These were plated out and isolated. Pure cultures of potential antagonists were produced and then each organism was screened individually to assess its potential as a biocontrol agent as outlined by Wilson and Wisniewski (1989) and Hofstein *et al*. (1994).

Mechanism of Antagonism

Most antagonistic yeasts are efficient colonizers, even under adverse environmental conditions, as they utilize nutrients rapidly, produce extracellular materials that enhance their survival on fruit surfaces and restrict

both colonization sites and flow of germination caused to fungal propagules (Dugan and Roberts, 1995). In order to optimize disease control, it is important to understand the mode of action of the antagonists so that these attributes can be utilized to improve performance. The antagonist activity can be expressed in a number of ways. The most common is antibiosis (production of metabolites such as pyrrolnitrin or iturins), attributed mainly to bacterial antagonists (Smilanick and Dennis-Arrue, 1992). Spadaro *et al.* (2002), in studies on *M. pulcherrima*, found that in the *in vitro* antagonism studies on different substrates, the yeast could produce some metabolites toxic to the pathogen, as distinct from the application of culture filtrates *in vivo*. Competition for nutrients and/or space is the major mechanism involved for *P. guilliermondii, C. laurentii, C. utilis, C. oleophila, D. hansenii* and several other yeasts employed as bioagents (Chalutz and Wilson, 1990; Arras, 1996; Arras *et al*., 1997; Spadaro *et al*., 2002; He *et al*., 2003; Chan and Tian, 2005; Zhang *et al*., 2005). Janisiewiez *et al.* (2000) developed a non-destructive method using tissue culture plates having a defusing membrane at the lower end of cylindrical inserts for *in vitro* study of competition for nutrients separated from the competition for space. Viable spores of the antagonist are necessary for efficient fungal management. The ability to prevent infection by pathogen was lost when the antagonist cells were mortal. It was also observed that competition for nutrients was not visible when a surplus of nutrients was available. Therefore, the nutritional environment available at the wound site may create a favourable microenvironment for antagonists to colonize, multiply and compete effectively (Zheng *et al*., 2004). The activity of an antagonist is dependent on the concentration of the antagonist: the higher the concentration, the more effective the control. The antagonist cell concentration of $10^6 – 10^8$ CFU/ml or more of *Candida* spp., *D. hansenii* and *Pantoea agglomerans* provided satisfactory levels of control (Droby *et al*., 1989; McLaughlin *et al*., 1990). However, different isolates of *M. pulcherrima* at 10^6 CFU/ml were not found to provide satisfactory levels of control against *B. cinerea* and *P. expansum* (Spadaro *et al*., 2002). While early studies indicated that nutrient competition and the fast growth rate of antagonists played a major role in biocontrol activity, subsequent studies indicated a much more complex interaction, such as direct interaction with the pathogen (Wisniewski *et al*., 1991; Spadaro *et al*., 2002), induced resistance in host tissue (Wilson *et al*., 1994; Droby *et al*., 2002) or a gamut of interactions between the antagonist, pathogen and commodity. *Pichia guilliermondii* US-7 (Droby *et al*., 1989) and *M. Pulcherrima* (Spadaro *et al*., 2002) exhibited nutrient competition along with direct parasitism against *B. cinerea* in apples. *Pichia membranefaciens* and *C. albidus* exhibited tenacious attachment with pathogen hyphae, along with secretion of extracellular lytic enzymes (Chan and Tian, 2005). Ultrastructural and cytochemical studies on yeast, *C. saitoana*, when co-cultivated with *B. cinerea*, showed cytological damage as papillae and protuberances in the cell wall and degeneration of the cytoplasm. It was also found to stimulate structural defence response in the host. Host cell walls were well preserved and displayed an intense and regular cellulose labelling pattern, as seen in transmission electron microscopy (El Ghaouth *et al*., 1998). Yeast cells are able to produce hydrolytic enzymes capable of attacking the cell walls of pathogens and extracellular polymers that appear to have antifungal activity. Yeast, *P. anomala* strain K, effective in the control of grey mould of apple, increased production of exo-b-1,3-glucanase threefold in the presence of cell wall preparations of *B. cinerea* in apple wounds. Higher b-1,3- glucanase and chitinase activity was also detected in apple wounds treated with strains of another antagonist, *A. pullulans*, effective in controlling various decays on apple, table grape and other fruits (Ippolito *et al*., 2000; Castoria *et al*., 2001). Yeast, *P. membranefaciens* and *C. albidus*, show b-1,3-glucanase and exo-chitinase activity in the presence of cell wall preparations of *R. stolonifer, M. fructicola* and *P. expansum* (Chan and Tian, 2005). Yeasts like *C. famata* are reported to control green mould due to induction of phytoalexins, scoparone and scopolectin (Arras, 1996). However, the role of enzymes and phytoalexins in biocontrol activity demands further investigation. Fajardo *et al.* (1998) reported differential induction of proteins in orange flavedo by biologically based elicitors. More recently, molecular approaches to examine the mode of action have been studied on the biocontrol agent. A transformation system for *C. oleophila* yeast produced yeast lines with either higher or lower levels

of a b-1,3-glucanase gene/enzyme expression compared to the wild type. Biocontrol activity did not differ between the different yeast lines, but the results did not rule out a role for this gene in biocontrol activity. It was also demonstrated that overexpression of a lytic peptide belonging to the defensin family of antimicrobial peptides in yeast could enhance biocontrol activity (Segal *et al*., 2002; Yehuda *et al*., 2003).

Integrated Disease Management

Since, biological agents alone are less capable of providing commercially acceptable levels of disease management, their integration with other control tactics is required to provide greater stability and effectiveness. It is also desirable that the use of antagonists must be compatible with current methods and storage practices which could otherwise cause a reduction in the effectiveness of antagonist strains. For biological control to be effective, use of antagonists must be compatible with other management methods. An effective biocontrol based on a mixture of several complementary and non-competitive antagonists has several advantages: apart from a wider spectrum of activity, they increase efficacy, are more reliable and allow reduction in application times and treatment costs. They also permit the combination of different genetic characteristics, minimizing the need for genetic engineering. In a study on apples, a broader spectrum of phytopathogens was controlled and less total biomass of the antagonist was needed to control decay (Janisiewiez, 1996) when a mixture of antagonists was applied. The mixtures are either paired at random or after screening, for minimum mutual niche overlap. To determine further compatibility of the strains selected, it is important to conduct coexistence studies using De Wit displacement series in fruit wounds (Wilson and Lindow, 1994). The best way of practical implementation is using in the mixture at least one antagonist which has been commercialized. Some exogenous substances, such as chitosan, amino acids, antibiotics, calcium salts and carbohydrates, have been studied to enhance the biocontrol capability of antagonists against fungal phytopathogens. Calcium chloride improved biological control of the yeast, *P. guilliermondii* (Droby *et al*., 1997). Combining 0.2% glycol chitosan with the antagonist, *C. saitoana*, was more effective in controlling green mould of oranges and lemons, caused by *P. digitatum*, and grey and blue moulds of apples than either treatment alone (El-Ghaouth *et al*., 2000a, b). In a recent study by the authors, a combination of chitosan and the yeast, *C. utilis*, was found effective in controlling postharvest pathogens on tomato (Sharma *et al*., 2006). The studies also showed that several yeast genera were compatible with low concentrations of chitosan and the protection afforded by this combination was superior to the stand-alone treatments. GRAS (generally recognized as safe) substances such as sodium carbonate, sodium bicarbonate and ethanol reduced conidial germination of *P. digitatum*, the causal agent of green mould of citrus. Ethanol at 10%, in combination with ethanol-resistant *S. cerevisiae* strains 1440 and 1749, reduced the incidence of grey mould decay on apples from more than 90% to close to 0%, respectively, whereas either treatment alone did not reduce decay. The same concentration of ethanol reduced green mould of lemons to less than 5% (Smilanick *et al*., 1995 and 1999). *A. pullulans*, in combination with calcium chloride or sodium bicarbonate, was found effective in controlling postharvest pathogens on sweet cherries (Ippolito *et al*., 1998). Pre-storage hot air treatment of apples reduced or eliminated blue mould decay caused by *P. expansum* and grey mould decay (Fallik *et al*., 1995). Heat also improved biocontrol with heat-tolerant yeasts when applied to apples up to 24 h after inoculation with the pathogen. The heat treatment alone provided little residual protection, but the residual protection provided by Ca and the antagonist in combination enhanced the control by heat. When antagonists were applied to apple wounds before heat treatment, the heat reduced populations of *P. syringae* and increased populations of the two heat-tolerant yeasts more than tenfold.

Challenges in Formulation development

In the early years, several yeast antagonists that had commercial potential were misidentified, such as strain US-7 of *C. guilliermondi*, which was misidentified originally as *D. hansenii*. This caused some confusion in the patenting process and emphasized the need to have at least two confirming identifications by reputable yeast taxonomists. It also emphasized the weakness of using physiological tests as the basis for making taxonomic determinations (McLaughlin *et al*., 1990). Also, few isolates of *C. Guillliermondii* were abandoned because they were

found to be pathogenic to humans. Potential biocontrol agents often have some signifi cant limitations: sensitivity to adverse environmental conditions such as extreme dryness, heat and cold, limited shelf life, limited biocontrol efficacy in situations where several pathogens are involved in decay development and an inability to control latent infections. For commercialization, several semi-commercial and commercial trials have to be conducted, for which large volumes of antagonist are required. The mass production of the bioagent by rapid, efficient and inexpensive fermentation of the antagonist is a key issue. Therefore, it is fundamental to find carbon and nitrogen sources that provide maximum biomass production at minimum cost, while maintaining biocontrol efficacy. Cheap and feasible industrial waste materials such as cottonseed meal, corn steep liquor, partially digested peptone, yeast extract, dry brewer's yeast, sucrose and molasses have been used as growth media for the multiplication of spores (Hofstein *et al.*, 1994; Costa *et al.*, 2001). Large-scale production of any yeast depends on the amount of technical information available on that specific strain, such as osmotolerance, temperature, oxygen requirements, optimum pH and optimum growth rate. Growth rate of yeast is very high, but lower than that of bacteria; longer fermentation durations pose the risk of yeast cultures becoming contaminated. Yeast is also sensitive to low pH (below three), which is used generally as a measure to check bacterial contamination because pH above five is favourable for bacteria that may contaminate yeast culture. Aeration of fermentors, to fulfil the oxygen requirement for maximum output, can also be a source of contamination during the early phases of production and, to prevent such contamination, other technologies must be used. The contaminants should be identified at each stage of production and quantified in the end product. Yeast fermentation is an exothermic process; therefore, the fermentation temperature can never be below ambient and, since yeasts appear sensitive to high temperatures (above 28°C), a cooling system more efficient than the evaporative system routinely used has to be employed. This, however, adds to the cost of production. A major obstacle to the commercialization of biocontrol agents is the development of a longer shelf life bearing stable product that retains bioactivity similar to that of fresh spores. Formulations can influence the survival and activity of biocontrol agents. An accurate formulation has a profound effect on the efficacy of a biocontrol agent, including its shelf life, ability to grow and survive after application, effectiveness in disease control, ease of operation and application and the cost (Fravel *et al.*, 1998). A biofungicide should be effective for at least 6 months, and preferably for 2 years (Pusey, 1994). This can be achieved by supplementing the yeast with protectants, carriers or additives. Alternatively, yeast can be conditioned during fermentation by using an emulsifier. Drying the product and maintenance in a dry environment or suspension in oil are common approaches. Products are available as wettable powder, as frozen cell concentrated pellets or as liquid formulations. It was found that freeze-dried spores were significantly less effective than fresh spores. The application of adjuvant can protect and stimulate the establishing of the antagonist on the host surface. Wetters not only make water spray stay on plants but, like oil carriers, they also enable organisms to reach otherwise inaccessible places such as depressions, stomata and lenticels, thereby improving the chances of establishing antagonists for disease control. Oil carriers are expensive, but formulations containing oils can enhance the reliability of biological control agents (Jones and Burges, 1998). One of the major limitations with biological disease control is the inconsistency in efficacy that is often observed when useful antagonists reach the stage of large-scale testing, and which can arise from a variety of causes reflecting the biological nature of the control microorganism. Essentially, the organism must first survive application and then retain activity in the environment of use throughout the period when active control is required, which may be several months for some pathogens. During this time, it must survive fluctuations in the physical environment and the action of the indigenous and competitive microbiota. The use of appropriate inoculum production, formulation and application technologies, together with quality control checks, should also help in this process. Nevertheless, even if reliable BCAs can be produced, they must still be easy to use and cost-effective or they will either never reach the marketplace or not be used by growers. By early 2000, there were two yeast based postharvest biological products available on the market: Aspire™ (*C. Oleophila* I-182) and YieldPlus™ (El-

Ghaouth and Wilson, 1997, 2002; Wilson and El-Ghaouth, 2002). The commercial development of Aspire by Ecogen-Israel Partnership Ltd, focused on the biocontrol of postharvest decays of citrus, mainly blue mould and green mould caused by *P. italicum* and *P. digitatum*, respectively, which invade through wounds after harvest. Throughout the course of developing Aspire™, considerable research went into finding methods to enhance the reliability and efficacy of the product and other selected antagonists as well. As a result, second generation biocontrol products were developed using a combination of natural products along with a yeast antagonist to address the poor ability. Research efforts led to the development of two new products whose main components consisted of the yeast antagonist, *C. saitoana*, and either a derivative of chitosan (Biocoat) or lysozyme (Biocure) (El Ghaouth *et al*., 2000a). Both compounds have been tested worldwide and have shown strong eradicant activity. Both products contain additional additives, such as sodium bicarbonate, to enhance efficacy and perform as well as the postharvest fungicides currently available.

CONCLUSIONS

Future niche areas of research should be focussed methods of exploring the biodiversity and enhancing the reliability and efficacy of selected antagonists. It should aim at finding additives or physical control methods that will act synergistically with the antagonist. This involves integrating the product with a low- risk level postharvest fungicide. It has been reported that physical treatments such as hot air, curing, hot water brushing and combinations of the above with pressure infiltration of calcium could also increase the efficacy of antagonists. Using mixtures of antagonists, or combining antagonists with specific nutrients or sugar analogues, is also suggested as an approach to increase efficacy. Genetic manipulation of antagonists is a field in its infancy. Current efforts are focused on developing efficient transformation procedures for yeast antagonists and inserting genes for tracking the antagonist in the environment rather than enhancing biocontrol (Yehuda *et al*., 2001). Other approaches could be: the insertion of the gene for amylase under the constitutive promoter in some BCAs to allow effective use of the fruit carposphere starch; biocontrol strains with a higher capability to exploit the nitrogen compounds present or with a higher transport or metabolism rate of the limiting factor can be developed, because nitrogen is often a limiting substance when the biocontrol mechanism of action is competition for nutrients; and use of mutants that use new substrates, not metabolized by the pathogen, to provide a nutritional advantage or attempt to obtain strains resistant to phenolic compounds (Bizeau *et al*., 1989). Biological control of plant diseases in general and on fruit after harvest in particular is a niche market, with a relatively small profit potential. However, it is clear that the stage is set for biological control agents to play a greater part in agriculture and horticulture. This approach undoubtedly would encourage environmentally desirable products that are desired by the public to reach the marketplace rapidly.

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