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ORIGINAL ARTICLES

Influence of Some Fungicides Alternatives on The Growth Viability of Bacterial and Yeast Bio-agents *In Vitro*

M.M. Abdel-Kader and N.S. El-Mougy, R.S. El-Mohamady, N.G. El-Gammal, E.I. Embaby

Plant Pathology Department, National Research Centre, Dokki 12622, Giza, Egypt.

ABSTRACT

The effect of plant resistance inducers, some essential oils and plant extracts on the viability of bio-agents, *Bacillus subtilis*, *Pseudomonas fluorescens* and *Saccharomyces cerevisiae* was evaluated *In vitro*. Plant resistance inducers, *i.e*. Potassium mono hydrogen phosphate salt and Calcium chloride, mixture of Humic & Folic acids (AF) were tested. Plant extracts of three plant leaves, *i.e*. Halfa Bar (*Cymbopogon Proximus*); Ginger (*Zingiber officinale*) and Bay laurel (*Laurus nobilis*) were tested. Commercial essential oils of Cinnamon, Clove and Thyme were also used in the present work. The obtained results revealed that *P. fluorescens* was highly affected with different tested concentrations of plant resistance inducers followed by *B. subtilis* and *S. serevisiae,* respectively. Regarding the inhibitor effect of tested plant extracts, *B. subtilis* showed the highest sensitivity against tested concentrations of Ginger extract followed by *P. fluorescens* and *S. cerevisiae,* respectively. Moreover, another feature was observed with *S. cerevisiae* that it showed more sensitivity than the other two bacterial isolates against Bay laurel and Ginger extracts concentrations. *In vitro* tests revealed that the used essential oils had an inhibitory effect against viability of tested bacterial and yeast. The reduction in colony formation of either bacteria or yeast was increased as concentration of tested essential oils was increased to reach its maximum at the highest tested concentration. The obtained results in the present study lead to suggest that the applied dose of such fungicides alternatives should be reviewed when combined with biocontrol agents in integrated control programs.

Key words: soil borne pathogenic fungi, viability of antagonistic bacteria and yeast, plant resistance inducers, some essential oils and plant extracts**,** growth viability.

Introduction

Plant pathogens have caused an almost 20% reduction in the principal food and cash crops worldwide (Oerke *et al*., 1994). Root rot caused by soil borne pathogenic fungi is one of the most serious diseases affected several cultivated plants worldwide. It results in poor production, poor quality, poor milling returns and reduced agriculture income. This has a negative impact on the livelihood of farmers. Fungal disease control is achieved through the use of fungicides which is hazardous and toxic to both people and domestic animals and leads to environmental pollution.

Therefore, a more balanced, cost effective and eco-friendly approach must be implemented and adopted farmers. In order to overcome such hazardous control strategies, scientists, researchers from all over the world paid more attention towards the development of alternative methods which are, by definition, safe in the environment, non-toxic to humans and animals and are rapidly biodegradable.

Such strategy is use of Biocontrol agents (Sivan, 1987; Punja and Utkhede, 2004) to control fungal plant diseases as well as other fungicides alternatives, *i.e*. plant resistance inducers (Punja and Grogan, 1982; Smilanick *et al*., 1999; El-Gamal *et al*., 2006; Abdel-Kareem, 2007; Ragab *et al*., 2009); essential oils (Sivropoulou *et al*., 1995 & 1997; Hammer *et al*., 1999) and plant extracts (Ashrafuzzaman, and Hossain, 1992; Mau *et al*., 2001; Ranaware *et al*., 2010).

The objectives of the present study were to evaluate the potential effect of some plant resistance inducers, essential oils and aqueous extract of some medicinal plants on the viability of bacterial and yeast bio-control agents in order to improve the efficacy of biological control when combined with such fungicides alternatives approach.

Materials and Methods

The inhibitory effect of plant resistance inducers, some essential oils and plant extracts on the viability of bio-agents, Bacteria and yeast was evaluated *in vitro*.

Corresponding Author: N.S. El-Mougy, Plant Pathol. Dept., National Research Centre, Giza, Egypt.
E-mail: nehal_nrc@yahoo.com

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Tested Bacteria and Yeast:

Bacillus subtilis, *Pseudomonas fluorescens* and *Saccharomyces cerevisiae.*

Tested Materials:

- Three concentrations of each of plant resistance inducers, Potassium and Sodium bicarbonates, Potassium mono hydrogen phosphate salt (K_2HPO_4) and Calcium chloride ($CaCl_2$), *i.e*. 1; 2 and 4% (w:v) or 0.2, 0.4, and 0.6 % for a mixture of Humic & Folic acids (AF) were tested.
- Commercial essential oils of Cinnamon (*a.i*. cinnamic, aldehyde, 70-85%)**,** Clove (*a.i*. eugenol, 90–95%) and Thyme (*a.i*. Thymol, 60%) were also used in the present work. Essential oils used in the study were obtained from Chemical Industrial Development Company (CID), Egypt. For each of the essential oil, three concentrations, *i.e*. 0.25, 0.5 and 1% were prepared and tested.
- Plant extracts of three plant leaves, *i.e*. Halfa Bar (*Cymbopogon Proximus*); Ginger (*Zingiber officinale*) and Bay laurel (*Laurus nobilis*) were tested. The plant materials kindly obtained from Medicinal and Aromatic Plants Research Department, NRC, Egypt. The materials were washed with distilled water and dried in shade.

The dried plant materials were then finely grinded to powder. Fifty grams of each plant material in powder form was homogenized by laboratory blender in 200 ml of ethanol (96%) and distilled water (20:80, v:v) for 10 min, then left in dark glass bottles for 72 h for tissue maceration. The extracts were filtered through thin cheesecloth sheets. The final extracts were collected separately in other dark glass bottles and exposed to 60°C in water bath for 30 min for ethanol evaporation. The collected extracts were then stored in a refrigerator at 5°C until needed. The extracts were added to sterilized PDA flasks before solidifying to obtain the proposed concentrations of 1, 2 and 4% (v/v) and Bay laurel at concentrations of 1.0, 2.0 and 4.0% (v:v)

In Vitro Laboratory Tests:

The antagonistic bacteria were grown on nutrient broth medium while yeast was grown on NYDB medium (Abd-Alla *et al*., 2009). All tested bacteria and yeast incubated in a rotary shaker at 200 rpm for 24 h at 28 ± 2^0C. The bacterial and yeast cells were harvested by centrifugation at 6,000 rpm for 10 min, washed twice with 0.05 M phosphate buffer at pH 7.0, and re- suspended in distilled water. The concentrations of both bacteria and yeast cells in the suspensions were adjusted to $1x10^4$ cells per milliliter (Cfu / mL) with the aid of a haemacytometer slide.

The inhibitory effect of tested materials on colony formed by antagonistic bacteria and yeast isolates was assayed in Nutrient and NYPD broth media using a modified method of Piano *et al*., (1997). Aliquots of 1000 µL of the bacterial or yeast cells suspension ($1x10^4$) were transferred to glass tubes (180x16 mm) containing 9 mL sterilized distilled water, then the tested materials were added individually to each tube to achieve the proposed concentration. All tubes were left for 12 h, then shaking well using Vortex for 5 min. One ml of each test tube was dispensed into Petri dish and about 20 mL of semi-solidifying sterilized agar medium (Nutrient or NYPD) were poured into the inoculated dishes and rotated gently to ensure equal distribution of the bacterial or yeast inocula. Control check treatment was the bacteria or yeast cells suspension free from tested materials. All dishes were incubated for 72 h at 28 ± 2^0C and then examined. Average percent of bacteria and yeast isolates formed colonies was calculated by comparing with their counts in check treatment. All treatments consisted of ten replicates, and experiments were repeated three times.

Statistical Analysis:

All experiments were set up in a complete randomized design. One-way ANOVA was used to analyze differences between antagonistic inhibitor effect and linear growth of pathogenic fungi *in vitro.* A general linear model option of the analysis system SAS (SAS Institute Inc. 1996) was used to perform the ANOVA. Duncan's multiple range test at $P < 0.05$ level was used for means separation (Winer 1971).

Results and Discussion

The effect of plant resistance inducers, some essential oils and plant extracts on the viability of bio-agents, *Bacillus subtilis*, *Pseudomonas fluorescens* and *Saccharomyces cerevisiae* was evaluated *in vitro.*

The viability of tested bio-agents, bacterial and yeast isolates affected significantly with plant resistance inducers. *Pseudomonas fluorescens* was highly affected with different tested concentrations of plant resistance inducers followed by *B. subtilis* and *S. cerevisiae,* respectively. Represented data in Table (1) and Fig (1) showed that different concentrations of Calcium chloride had significant inhibitor effect on the viability of

bacterial and yeast cells. The highest concentration tested (4%) significantly reduced viability of *B. subtilis* (from 135.6 to 123.3, $1x10^4$ cfu/mL); *P. fluorescens* (from 164.4 to 116.1, $1x10^4$ cfu/mL) and *S. serevisiae* (from 130.2 to 106.2, $1x10^4$ cfu/mL), respectively. Similar results are also reported in this regards against several microorganisms, Calcium chloride suppresses the bitter rot pathogens, *Colletotricum gloeosporioides* and *C. acutatum* (Biggs, 1999). He added that suppressive include reduced germ tube growth, reduced mycelia growth *in vitro.* Also, reduction in spore germination of *Penicillium digitatum* exposed to calcium chloride has been observed (Droby *et al*., 1997), as well as reduced germination and germ tube of *P. expansum* and *Botrytis cinerea* (Wisniewski *et al*., 1995). Calcium salts also have been shown to reduce mycelial growth of *Alternaria, Monilinia fructicola* and *Leucostoma persoonii* the causal fungi of apple peach fruit decay *in vitro* (Biggs and Peterson, 1990; Biggs *et al.,* 1993 & 1994). Similar trend was also noticed concerning the inhibitor effect of the mixture Humic and Folic acids on viability of *B. subtilis*, *P. fluorescens* and *S. cerevisiae in vitro* conditions. Data in Table (1) and Fig (1) showed significant gradual decrease in bacterial and yeast viability with increasing concentrations of Humic and Folic acids mixture. Concentration of 0.6% significantly reduced viability of *B. subtilis* and *P. fluorescens* by 15.4 and 31.2%, respectively (Fig. 2). The tested yeast (*S. serevisiae*) showed similar response with different concentrations of Humic and Folic acids mixture. Viability of *S. serevisiae* was lesser significantly reduced by 13.8% at 0.6% concentration of Humic and Folic acids mixture. It was also noticed that the other two lower concentrations used (1, 2% Calcium chloride) and (0.2, 0.4% Humic and Folic acids mixture) showed no significant reduction, between each other, on bacterial viability, although they were significantly differed from control check treatment. In this concern several investigators reported the beneficial effect of Humic and Folic acids as plant growth stimulants as well as low effect on the microorganisms. Vallini *et al*., (1993) have reported the increase in the number of total bacteria and actinomycetes in the rhizosphere of laurel caused by humic acid treatments. They added that changes in the metabolism process of plants may induce a resistance to certain phytopathogens. Similarly, changes in the microbial activity may negatively affect certain soilborne pathogens, and changes in physicochemical and chemical properties of the soil after amendment addition was also unfavorable to pathogens (Huang *et al*., 2006). Also, Abdel-Kareem *et al*., (2009) reported that all tested concentrations of humic acid had no inhibitory effect on *Alternaria solani in vitro*.

In the present study, Potassium, Sodium bicarbonates and Potassium mono-hydrogen phosphate at concentrations of 1, 2 and 4% were tested against each of *B. subtilis, P. fluorescence* and *S. cerevisiae.* Data in Table (1) and Fig (1) showed the reduction in the colony formation of antagonistic bacteria and yeast in response to different concentrations of these salts. Reduction in viability of tested bacteria and yeast isolates, significantly increase as the tested chemical concentrations were also increased to reach its maximum reduction at concentration 4% of all tested chemicals.

Moreover, illustrated data in Fig. (1) showed that *P. fluorescens* had the highest significant sensitive response against concentrations of tested Sodium bicarbonate (Fig. 1) whereas their viability were reduced by 28.7, 40.2 and 44.4% at concentrations of 1, 2 and 4% followed by *S. cerevisiae* (18.1, 38.2 and 38.4%) and *B. subtilis* (7.6, 22.3 and 29.6%), respectively.

Table 1: Effect of some plant resistance inducers on the viability of antagonistic bacteria and yeast *in vitro.*

chemicals	Concentration (%)	Antagonistic bacteria and yeast		
		B. subtilis	*P. fluorescence*	*S. sevisease*
Control	0	135.6* a	164.4 a	130.2 a
Humic and Folic acids mixture	0.2	128.4 ab	124.2 b	125.6 b
	0.4	125.1 b	120.9 b	114.6 c
	0.6	114.6 c	113.1 c	112.2 c
Calcium chloride	1	130.2 ab	126.3 b	118.2 c
	2	125.4 b	120.8 b	110.8 cd
	4	123.3 b	116.1 c	106.2 cd
Sodium bicarbonate	1	125.2 b	117.2 c	106.6 cd
	2	105.3 cd	98.3 d	80.4 e
	4	95.4 de	91.4 de	80.2 e
Potassium bicarbonate	1	118.3 c	112.2 c	100.4 d
	2	98.2 de	98 3 de	76.6 f
	4	83.7 e	84.6 e	60.4 g
Potassium mono-hydrogen phosphate	1	40.8 h	46.3 h	100.2 d
	2	32.3 i	43.2 h	82.7 e
	4	18.6 k	25.7 j	72.4 f

Mean values within columns followed by the same letter are not significantly different ($P \leq 0.05$).
* Each figure represent bacterial or yeast average counts estimated as $1x10^4$ cfu/mL

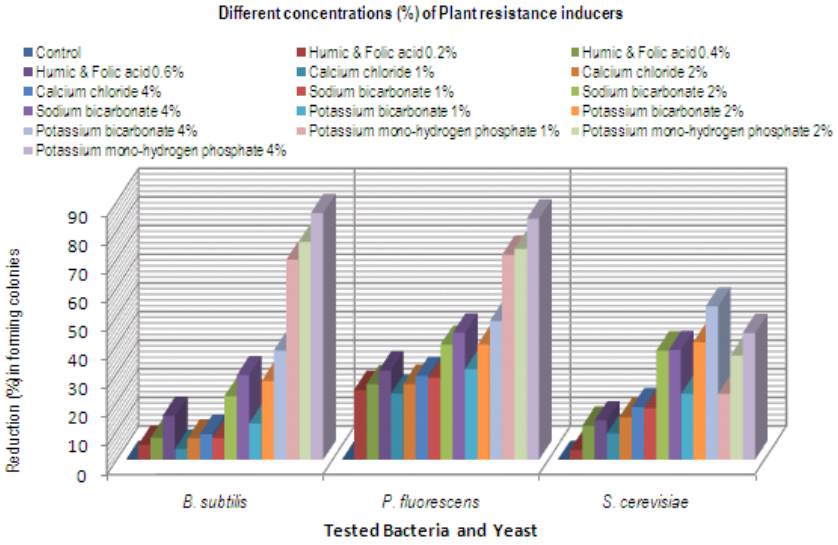

Fig. 1: Reduction (%) in viability of antagonistic bacteria and yeast in response to different concentrations of some plant resistance inducers *in vitro*.

Reduction in numbers of bacterial colony formation calculated relatively to formed bacterial colonies in control (free of plant resistance inducers)

Similar trend was also observed when Potassium bicarbonate added to the growth medium at the concentration of 1%. This arrangement was differed in response to Potassium bicarbonate where *S. cerevisiae* showed the highest sensitivity followed by *P. fluorescens* and *B. subtilis* at concentrations of 2 and 4%. Concentrations of Potassium mono-hydrogen phosphate at 2 and 4% showed the highest inhibitor effect against viability of *B. subtilis* (76.1, 86.2%) followed by *P. fluorescens* (73.7, 84.3%) and *S. cerevisiae* (36.4, 44.3%), respectively. In this regards, many investigators studied the influence of various salts on microorganisms. There was a considerable interest in the use of sodium bicarbonate ($NaHCO_3$) and potassium bicarbonate ($KHCO_3$) for controlling various fungal diseases in plants (Karabulut *et al.* 2003; Smilanick *et al.* 2006). Bicarbonates are widely used in the food industry (Lindsay 1985) and were found to suppress several fungal diseases of cucumber plants (Ziv and Zitter 1992). Spraying plants with $NaHCO_3$ solution provided good control of several plant diseases (Horst *et al.* 1992; Arimoto *et al.* 1997; Palmer *et al.*1997; Janisiewicz and Peterson 2005). Also, spraying with KHCO3 solution provided the most effective protection against plant diseases (Smilanick *et al.* 1999, 2006). Sodium or potassium bicarbonate combined with oils were effective in controlling plant diseases (Horst *et al.* 1992; Ziv and Zitter 1992). Furthermore, the antimicrobial activity of sodium carbonate and sodium bicarbonate has been described *in vitro* (Corral *et al*., 1988; Curran *et al*., 1990; Smilanick *et al*., 1999). Also, exposure of *F. solani* var. *coeruleum* conidia to aluminum acetate, potassium sorbate, sodium benzoate, sodium metabisulfite or trisodium phosphate at 0.2 M resulted in 100% mortality of the conidia after 1 h while aluminum chloride and aluminum lactate caused 100% mortality after an exposure of 24 h. (Mills *et al*., 2004; Melane *et al*., 2009).

Regarding the inhibition effect of Cinnamon, Clove and Thyme essential oils at different concentrations on viability of *B. subtilis, P. fluorescens* and *S. cerevisiae.* Obtained results are presented in Table (2) and Fig (2). *In vitro* tests revealed that the used essential oils had an inhibitory effect against viability of tested bacterial and yeast. The reduction in colony formation of either bacteria or yeast was increased as concentration of tested essential oils was increased to reach its maximum at concentration (1.0%). It was also observed that, concentration of 0.25% of all tested essential oils had no effect against viability of tested bacterial and yeast isolates. Data also showed that *P. fluorescens* was the most sensitive microorganism to the inhibitory effect of tested concentrations of Cinnamon, Clove and Thyme essential oils followed by *B. subtilis* and *S. cerevisiae* in respective order. Also, both cinnamon and Thyme oils had superior inhibitor effect against the viability of tested bio-agents comparing with clove essential oil.

Plant extract of Halfa Bar (*Cymbopogon Proximus*), Ginger (*Zingiber officinale*) and Bay laurel (*Laurus nobilis*) at concentrations of 1, 2 and 4% were evaluated for their inhibitory effect against viability of *P. fluorescens, B. subtilis* and *S. cerevisiae in vitro* conditions. Data in Table (3) and Fig (3) showed that the inhibitor effect of used plant extracts against viability of tested bacteria and yeast isolates increased by increasing their concentrations. Data also revealed that the tested microorganisms varied among each other in their response against the tested plant extracts. In this regard, illustrated data in Fig. (3) *B. subtilis* showed the

highest sensitivity against tested concentrations of Ginger extract followed by *P. fluorescens* and *S. cerevisiae*, respectively. Moreover, another feature was observed with *S. sevisease* that it showed more sensitivity than the other two bacterial isolates against Bay laurel and Ginger extracts concentrations. The highest reduction in colony formation of *P. fluorescens*, *B. subtilis* and *S. cerevisiae* was 51.7, 51.8 and 67.4% at concentration (4%) of Bay laurel extract. Meanwhile, growth reduction was recorded (48.0, 48.7 and 36.5%) as well as (37.0, 36.6 and 50.5%) at concentration (4%) of Halfa Bar and Ginger extracts, in respective order.

Table 2: Effect of some essential oils on the viability of antagonistic bacteria and yeast *in vitro*.

Essential oils	Concentrations (%)	Antagonistic bacteria and yeast		
		B. subtilis	*P. fluorescens*	*S. cerevisiae*
		No. of colony (10^4/ml)		
Control	0.0	135.6* a	164.4 a	130.2 a
Cinnamon	0.25	135.3 a	164.1 a	130.1 a
	0.5	115.4 b	104.3 b	104.2 b
	1.0	83.6 c	89.6 c	100.3 bc
Clove	0.25	135.3 a	164.2 a	130.2 a
	0.5	122.2 b	132.3 b	120.6 b
	1.0	102.7 c	118.2 d	118.4 bc
Thyme	0.25	135.1 a	164.0 a	130.3 a
	0.5	120.2 b	112.2 b	112.1 b
	1.0	89.6 c	96.4 c	102.2 bc

Mean values within columns followed by the same letter are not significantly different ($P \leq 0.05$).
* Each figure represent bacterial or yeast count estimated as $1x10^4$ cfu/mL

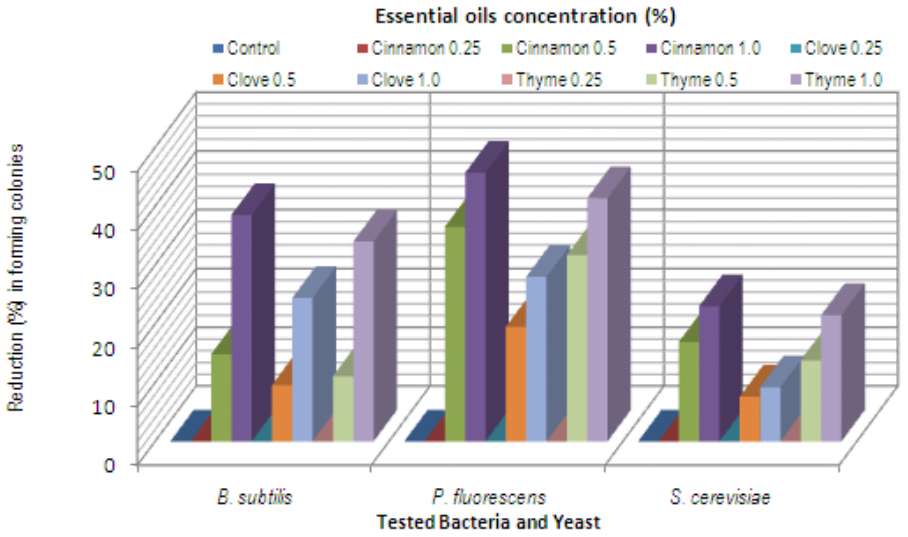

Fig. 2: Reduction in the colony formation of some antagonistic bacteria and yeast in response to different concentrations of essential oils *in vitro.*

Reduction in numbers of bacterial colony formation calculated relatively to formed bacterial colonies in control (free of essential oils)

Table 3: Effect of some plant extracts on the viability of antagonistic bacteria and yeast *in vitro.*

Plant extracts	concentration	Antagonistic bacteria and yeast		
		B. subtilis	*P. fluorescens*	*S. cerevisiae*
		No. of colony (10^4/ml)		
control	0.0	135.6* a	164.4 a	130.2 a
Halfa Bar *Cymbopogon Proximus*	1	112.3 ab	122.2 ab	124.2 a
	2	88.4 bc	92.4 b	108.3 b
	4	70.4 d	84.2 c	82.6 c
Ginger *Zingiber officinale*	1	124.2 a	154.3 a	104.1 b
	2	98.3 b	118.2 ab	96.3 c
	4	85.4 bc	104.2 ab	64.4 d
bay laurel *Laurus nobilis*	1	108.2 ab	99.3 b	96.2 c
	2	84.4 c	82.4 c	72.4 d
	4	65.3 e	79.3 cd	42.4 e

Mean values within columns for each organism followed by the same letter are not significantly different ($P \leq 0.05$).
* Each figure represent bacterial or yeast count estimated as $1x10^4$ cfu/mL.

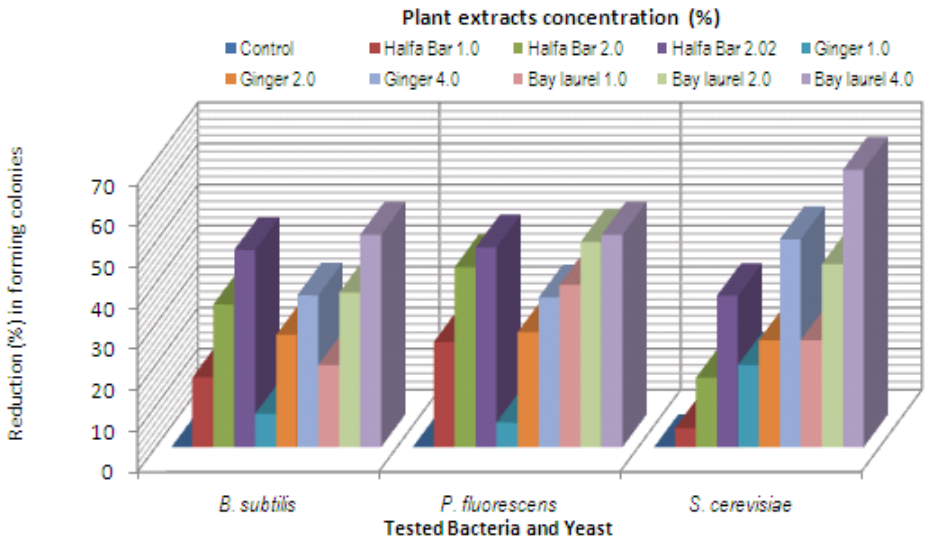

Fig. 3: Reduction in the colony formation of some antagonistic bacteria and yeast in response to different concentrations of plant extracts *in vitro*

Reduction in numbers of bacterial colony formation calculated relatively to formed bacterial colonies in control (free of plant extracts)

Many plants and plant products have been reported to possess pest control properties (Grayer and Harborne, 1994). Although much of the literature on natural products in the agricultural field concerns insect control, a smaller but emerging body of papers reports that plant extracts and plant essential oils are effective antimicrobials against food and grain storage fungi (Arras *et al.,* 1993; Mishra *et al*., 1994; Paster *et al*., 1995; Wilson et al., 1997; Montes-Belmont and Carvajal, 1998), foliar pathogens (Rao *et al*., 1992; Lawson and Kennedy, 1998), and soilborne fungi (Kishore *et al*., 1982; Smilanick *et al*., 1993; Pandey and Dubey, 1994; Muller-Riebau *et al*., 1995; Bianchi *et al*., 1997). Consideration will need to be given to the mechanism of the interaction of the product with the pathogen population and the host plant. Many plant extracts have volatile components, essential oils, and so on. Preliminary data suggest that some of these extracts and essential oils are capable of pathogen growth inhibition *in vitro* when tested so that only volatiles interact with the pathogen, while others only inhibit the pathogen when in direct contact (J. H. Bowers and J. C. Locke, *unpublished*). Information of this type is important as one tries to develop a delivery system that utilizes the extracts' physical and chemical properties.

Initial experiments in the greenhouse to test this strategy have had favorable results, where the addition of a biological control agent in combination with plant resistance inducers, some essential oils and plant extracts could be resulted in increased symptomless plant stand over the biological agent. Further research in this area has the potential to extend the usefulness of natural plant products and other bio-pesticides in crop production systems.

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