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RESEARCH ARTICLE :

Probiotics: A new approach for post harvest disease management and quality retension in grapes

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SUMMARY : Use of probiotics for the control of post-harvest diseases has opened new avenue in the management of post-harvest plant pathogens and organic farming. Such probiotics has never been used in the crop protection programme in agriculture. The post-harvest pathogens particularly *Rhizopus*, *Aspergillus*, *Penicillium* and *Alternaria* can be checked as a post-harvest pathogen by sprays of probiotics under natural field conditions when the loads of inoculums of these post-harvest pathogens are less in fruits. Application of probiotic as pre-harvest field spray helps to manage the post-harvest disease. The pre-harvest field application of commercial probiotics Sporocheck and MPKV probiotics has beneficial effect for management of post-harvest pathogens *Aspergillus*. Commercial Probiotics Darolac and Sacro was not effective in the control of *Aspergillus* infection. For the control of *Rhizopus* pathogen MPKV probiotics was effective as compared to commercial probiotics. similarly commercial probiotics Darolac, Sporocheck and MPKV probiotics was effective to inhibit the growth of post-harvest pathogens *Penicillium* and *Alternaria* on the harvested fruits (having natural load of pathogen inoculums) the efficacy of probiotics against post-harvest pathogens, indicated that probiotics can be used in the management of post harvest disease and there can form an integral part of organic farming system and keeps the grape berries fresh. As the probiotics are consumed orally and beneficial to the human health their presence on the consumable fruits will not have any harmful affect on the human. As the probiotics are known to control pathogenic infections in human and are safe microbial formulations, there use to control post harvest pathogen was studied.

KEY WORDS :
Probiotics, Post harvest pathogens, Disease management

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BACKGROUND AND OBJECTIVES

A fruit forms an important component of human diet as these are rich sources of minerals, vitamins, aminoacids, anthocyanidins and immune system boosters. Among various fruits, grape berries are a high priced fruits in India. In India, grape is cultivated over an area of 1.1 lakh ha with a productivity of 11.1 MT/ha (Anonymous, 2011) and total production of 12.35 million MT. Maharashtra state occupies 90,000 ha area with production of one million MT and ranks first in production and productivity with 62.7 per cent of countries total production.

Besides occurrence of regular diseases in grape orchards, post-harvest pathogens also infect the grape bunches in the gardens during humid climatic conditions, and in transit and

storage. The post-harvest losses of grapes in developing countries have been estimated upto 27 per cent (Steppe, 1976) and possibly the greatest single cause of post-harvest losses is caused by microorganisms. This usually occurs from initial infection by one or more specific pathogens during cultivation followed by secondary infection by a broad spectrum of saprophytes on the dead tissues during transit and storage. During storage, a number of fungi are known to cause spoilage of fruits. Under tropical conditions considerable losses are occurred due to rots caused by *Rhizopus* spp., *Penicillium* spp., *Aspergillus* spp., *Botrytis cinerea*, *Botryodiplodia* spp., *Bipolaris* spp., *Curvularia* spp., *Fusarium* spp., and are becoming limiting factor for successful fruit harvest management (Thomas, 1986). The total estimated losses of fruits and vegetables in India due to inadequate post-harvest handling, transportation and storage are reported to the extent of 20-25 per cent (Singh, 1981). These losses in terms of monetary value are more than 1.5 billion dollars annually.

Post-harvest losses caused by micro-organism accounts for millions of dollars in perishable produce every year (Narayana Swamy, 2005). To minimize these losses and the produce, it is very necessary to control the post-harvest diseases. Post-harvest diseases can be controlled by biological, chemical and physical treatments (Mehrotra *et al.*, 1998). These includes the use of bioagents and botanicals as biological treatments while chemical treatments includes use of antibiotics, fungicides, oils and as physical treatments vapor emitting compounds.

The post-harvest diseases minimized by application of therapeutical fungicides as pre-harvest treatment under field conditions, posses a serious threat of residues of these fungicidal chemicals on consumable product and therefore their use is being restricted worldwide. Therefore a novel method of biocontrol of these post-harvest diseases is a need of the day to save our produce. These biocontrol agents should be safe for human, as in case of Probiotics.

Probiotics are defined as the micro organisms which are beneficial in the human health (Nichols, 2007). These are orally administered or given in food supplements and are present in the gastrointestinal tract of human beings. These are also used for the health benefits of animals, birds and fishes (Sharma and Mamta, 2007 and Gatesoupe, 2008). Probiotics occur naturally in the fermented food product such as yoghurt, kefir, sauerkraut, cabbage kimchee and soybean-based miso and natto. Numerous health benefits have been attributed to probiotics including effects on gastrointestinal tract functions and diseases, immune functions, hyperlipidemia, hypertension and allergic conditions. These also enhance the recovery from fatigue and improved the immune function (Nichols, 2007).

The probiotic supplements includes bacteria like *Lactobacillus rhamnosus, Bifidobacterium longum, L. salivarius, L. plantarum, L. paracasei, B. lactis type* and *Streptococcus facealis, Clostridium butyricum* and *Bacillus masentericus* and some edible yeast species. As the probiotics are consumed orally and beneficial to the human health their presence on the consumable fruits will not have any harmful affect on the human. As the probiotics are known to control pathogenic infections in human and are safe microbial formulations, there use to control post harvest fruit pathogen was studied.

RESOURCES AND METHODS

Isolation of causal organism :

The collected disease specimens from grape berries were surface sterilized with 1 per cent sodium hypochloride solution for 2-3 minutes and partly diseased portion with some partly healthy portion was cut with the help of sterilized scissors under aseptic conditions in laminar air flow and kept on potato dextrose media plates. The inoculated petriplates were then incubated at 25±2°C for 3 days. The fungal growth obtained was purified by mycelial tip isolation method and further identified upto genus level based on their characteristics.

Commercial probiotics:

Sacro (contains Lyophilized *saccharomyces boulardi*), *Darolac* (contains *L. acidophilus, L. rhamnosus, B. longum* and *S. boulardi)* and *Sporocheck* (contains *Streptomyces faecalis, Clostridium butyricum, Bacillus mesentaricus and Lactic acid bacillus* (*Lactobacillus sporogenes*) available in the market (Plate A).

Isolation and culturing of commercial probiotics:

The commercial sachets of Sacro, Darolac and Sporocheck probiotics acquired from market was used for isolation and culturing of probiotics. The content of sachet was suspended in 10 ml sterile distilled water under aseptic condition and allowed to stand for 30 minutes. A

loopful of suspension was streaked on malt extract agar medium for isolation of yeast and on nutrient sucrose agar medium for isolation of bacteria. The plates were incubated at 28±2ºC for two days and growth of the probiotic micro-organisms were observed. The growth of probiotic isolates contained yeast cultures and bacterial growth.

Plate A : Commercial probiotic sachets used in the experiments

MPKV probiotics :

Probiotics were isolated from respective probiotic sample (Probiotic I, a edile yeast culture isolated from dhokla material; probiotic II, a edible yeast culture isolated from dosa material; probiotic III, a edible yeast culture isolated from bajra flour; probiotic IV, a edible yeast culture isolated from jowar flour and probiotic lactobacillus culture isolated from curd.

Isolation and formulation of MPKV probiotics :

Isolation of edible yeast :

Fermented samples of raw dosa and dhokla material were subjected from isolation of edible yeast on malt extract medium by streak plate method. A loopful of material was streaked from isolation samples on yeast isolation medium in Petri plates and inoculated plates were incubated in BOD at 28 ± 2ºC for 2 days for appearance of yeast colonies.

For isolation of edible yeast from cereal grain flours, the flour of jowar grain /bajra grain was suspended in distilled water (25g flour in 100 ml distilled water) and incubated at 28±2ºC in BOD incubator for three days to start the fermentation due to grain yeast. A loopful of suspension from this incubated and fermented flour sample was streaked on yeast medium and the plates were incubated for two days at 28±2ºC for appearance of yeast colonies. The isolated yeast colonies in the plates were further purified by streak plate method and single colonies thus obtained were maintained as pure cultures of the yeast as probiotics.

Isolation of *Lactobacillus :*

One loopful of curd taken from curd sample was streaked on the sterilized Nutrient agar sucrose (NAS) medium in Petri plates. The inoculated plates were incubated in BOD at 28 ± 2ºC for 2 days for observation of bacterial colonies. The isolated bacterial colonies in the plates were further purified by streak plate method and single colonies thus obtained were maintained as pure cultures of the bacteria (*Lactobacillus*) as Probiotic.

Morphological characterization of MPKV yeast:

The edible yeast isolated from dosa material, dhokla material, bajra flour and jowar flour was characterized for differences among them, if any, on the basis of cultural growth, microscopic observations, NaCl tolerance test and utilization of sugar concentrations.

Cultural growth :

The growth of the yeast culture *viz*., fluppy/raised/ suppressed with the colour of yeast colonies *viz*., dull creamy grey/ pale yellowish was recorded for the respective yeast isolate.

Microscopic studies :

The cells shape of yeast isolates *viz*., oblong/ cylindrical/elongated with budding habits and their size was recorded.

NaCl tolerance test :

The yeast isolates were tested for their NaCl tolerance. The malt extract agar medium having different concentration of NaCl *viz*., 0, 5, 10, 15 and 20 per cent were used. A loopful of yeast culture was streaked on the respective NaCl concentration tubes and was incubated in BOD incubator at 28±2ºC for 3 days to observe the growth of yeast on particular NaCl concentration to determine the NaCl tolerance limit.

Utilization of sugar concentration :

The malt extract agar medium having different concentration of sugar (sucrose) *viz*., 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 per cent were used. A loopful of yeast culture was streaked on the respective sugar

concentration tubes and were incubated in BOD incubator at 28±2ºC for 3 days to observe the growth of yeast on particular sugar (sucrose) concentration. The edible yeast isolated from dosa material, dhokla material, jowar grain flour and bajra grain flour were designated as yeast I, II, III and IV, respectively while Lactobacillus was designated as isolate V. The MPKV probiotic formulation contained the microbial cultures of all above isolates.

In vitro effect of probiotics on post harvest pathogens:

In one litre of distilled water 20 g sugar was added. In it one probiotic sachet of respective probiotic containing one gm of probiotic powder was added. This solution was incubated at 28 ± 2ºC in BOD for the growth of probiotic organisms for 3 days. The suspension thus obtained was used for field application. In case of MPKV probiotics one litre distilled water having 2 per cent sugar was taken. A growth of individual MPKV probiotic I, II, III, IV and V from a single tube was added to this solution and incubated at 28 ± 2ºC in BOD for the growth of probiotic organisms for 3 days. Two months old grape bunches was harvested and brought to the laboratory. There grape bunches were sprayed with probiotic solutions prepared as above and air dried. Such bunches were packed in package cardboard boxes and kept at ambient temperatures for observation on development of post harvest pathogen.

Field evaluation of probiotics for biocontrol efficacy against post harvest pathogens :

Two probiotics *viz*., Sacro and Darolac which were effective under *in vitro* test to simulate sugar brix in harvested grape berries were tested under field conditions for their efficacy. In one litre distilled water 20 g sugar was added. In it one probiotic sachet of respective probiotic containing one g of probiotic powder was added. This solution was incubated at 28 ± 2ºC in BOD for the growth of probiotic organisms for 3 days. Under field conditions the probiotic sprayed bunches were harvested after 8 days and 15 days of probiotic sprays. These grape bunches were packed in cardboard boxes along with control grape bunches (without probitotic spray). Observations were taken after 15 days of packing.

Preparation of probiotic solutions :

Commercial probiotic suspension for field application was prepared as fallows. In one litre distilled water 20 g sugar was added. In it one probiotic sachet containing one g of probiotic powder was added. This solution was incubated at 28 ± 2ºC in BOD for the growth of probiotic organisms for 3 days. The suspension thus obtained was used for field application. In case of MPKV probiotics one litre distilled water having 2 per cent sugar was taken. A growth of individual MPKV probiotic I, II, III, IV and V from a single tube was added to this solution and incubated at 28 ± 2ºC in BOD for the growth of probiotic organisms for 3 days. The suspension thus obtained was used for field application.

To study the antagonistic effect of probiotics on post-harvested fruits having natural post-harvest pathogens (*Rhizopus, Aspergillus, Alternaria* and *Pencillium*), the experiments were laid out *in vitro* conditions. The post-harvested fruits were brought to laboratory and sprayed with respective probiotics solutions. There were packed in their respective packing boxes and kept at room temperature for observation on development of post-harvest pathogens. In second experiment, the harvested fruits were brought to laboratory. These were washed with distilled water and dried with air by using air drier. These fruits were then spray inoculated with suspension of respective post-harvest pathogen and again air dried. The respective probiotic suspension was sprayed on these fruits and again air dried. The probiotic treated fruits were packed in their respective packing material as mentioned above and kept at room temperature. The fruits sprayed with pathogen but no probiotic served as control. The observation was taken upto one month for appearance of post-harvest pathogen. Yet in another experiment the fruits (grape berry bunches) under field conditions were sprayed with probiotics. There were then harvested after 8 days and 15 days of probiotic spray and were packed in cardboard grape packing boxes and kept at room temperature. Observations were recorded after 15 days for infection of post-harvest pathogens if any. The unsprayed fruits served as control.

OBSERVATIONS AND ANALYSIS

The results obtained from the present study as well as discussions have been summarized under following heads:

Growth characters of MPKV probiotics :

The growth characters of MPKV yeast Probiotic

(Table 1 and Plate 1) indicated that the probiotic isolate I and II has round, fluppy raised yeast colonies with dull creamy grey colony colour whereas the probiotic isolate-III had round suppressed yeast colonies with dull creamy grey colony colour and probiotic isolate-IV had round suppressed yeast colonies with pale yellowish colony colour. It was evident that the yeast of fermented dosa and dhokla material formed round fluppy raised colonies while that of bajra and jowar flour formed round suppressed colonies indicating that the dosa and dhokla yeast was same while bajra and jowar yeast was same. However the jowar and bajra yeast differed in their colony colour and therefore seems to be different.

Table 1 : Growth characteristic of MPKV yeast probiotics

Probiotic isolate	Colony character	Colour of colony
I	Round floppy raised	Dull creamy grey
II	Round floppy raised	Dull creamy grey
III	Round suppressed	Dull creamy grey
IV	Round suppressed	Pale yellowish

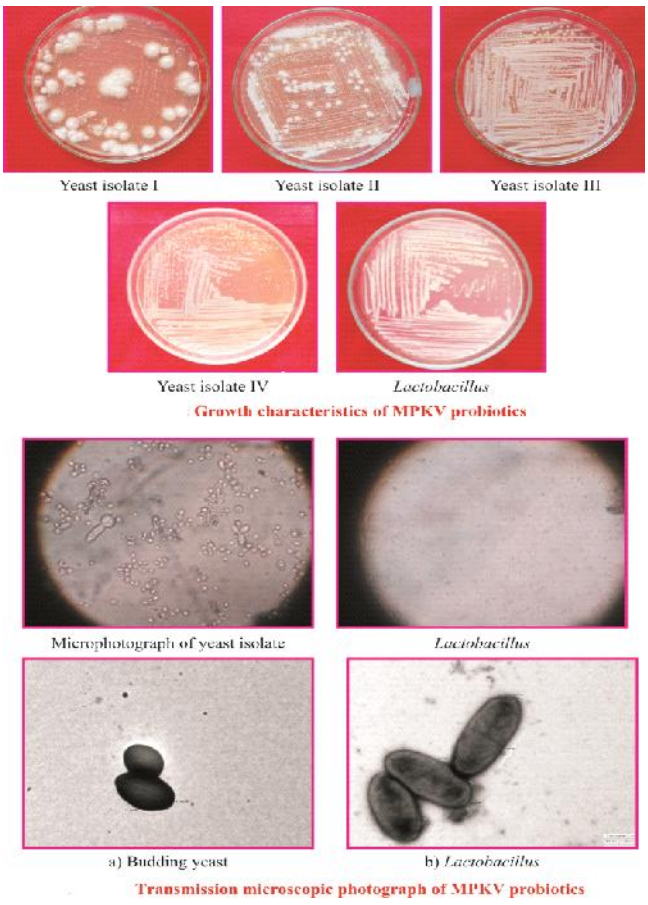

Plate 1 : Growth characteristic and microscopic characters of MPKV probiotics

Morphology of yeast isolate :

The morphological characters particularly yeast cell shape, size and budding habits were observed microscopically. The results (Table 2 and Plate 1) were indicated that the shape of yeast cells of probiotic – I and II was oblong while that of probiotic – III and IV it was elongated. It was evident that the fermented dosa and dhokla material had oblong cell yeast while in bajra and jowar flour it had elongated shape yeast. Further all the yeast isolates were variable in their cell size. The cell size of probiotic isolate- I was bigger (2.85 - 4.80 x 2.37 – 2.99) than other yeast isolates (1.05 – 1.15 x 0.91 – 0.92). All the yeast isolates had budding habits.

NaCl tolerance limit of MPKV yeast isolates:

The NaCl salt tolerance limit of all the four yeast isolate were studied by using salt concentration 0-20 per cent. The results (Table 3) indicated that all the 4 yeast isolates grew well on the medium having 10 per cent salt. At 15 and 20 per cent salt concentration there was no growth of yeast isolates.

The results indicated that the probiotic yeast can tolerate the NaCl salt concentration upto 10 per cent. On the basis of salt tolerance all 4 yeast isolates seems to be the same.

Growth of MPKV yeast isolates at different sugar concentrations :

The growth pattern of four MPKV yeast isolates in different sugar (sucrose) concentration was studied using the sucrose percentage from 0-50. The results (Table 4) indicated that all the four yeast isolates had full growth within 24 hours on the medium containing 20 per cent sucrose concentration whereas they produced scanty growth on 25-35 per cent concentration and no growth on 40-50 per cent sugar concentration. In 48 hours time the scanty growth of these isolates on 25-35 per cent sugar concentration turned into full growth whereas no growth on 40- 50 per cent turned into scanty growth and at 72 hours all the four yeast isolates got full growth on 50 per cent sugar concentration.

These results indicated that all the four yeast isolates could grow in the sucrose concentration upto 50 per cent. As the sugar concentration increases beyond 20 per cent, the period required for full growth also increases. On the basis of sugar utilization pattern all the 4 yeast isolates

seems to be same.

In vitro assessment of probiotics as biocontrol agent:

The results (Table 5a) indicated that the commercial probiotic Darolac, Sporocheck and MPKV probiotic was effective to inhibit/control the growth of post-harvest pathogen *Penicillium* and *Alternaria* only but not against the pathogen *Aspergillus* and *Rhizopus* under dual inoculam application. Commercial probiotic Sacro was not at all effective against any test pathogen.

The efficacy of their probiotics were also studied on naturally occurring post harvest pathogens on grape bunches. The result (Table 5b) indicated that the commercial probiotics Darolac and MPKV probiotics was effective against four post harvest pathogens *i.e.*, *Aspergillus, Rhizopus* and *Alternaria* while Sporocheck was effective against *Penicillium, Rhizopus* and *Alternaria*. The much efficacy of probiotics to control natural post harvest infection may be due to fact that the natural inoculum load of post harvest pathogen may be less to obtain cent per cent control.

Field performance of probiotics in management of natural post harvest fungi in grapes :

The results (Table 6a) indicated that commercial probiotic Sporocheck and MPKV probiotic were able to check the infection of *Aspergillus* fungus as there was no growth of *Aspergillus* in the probiotic sprayed bunches while it was present in the unsprayed fruit bunches. The probiotics Darolac and Sacro was not effective in the control of *Aspergillus* infection. For the control of *Rhizopus* pathogen MPKV probiotic was effective as compared to commercial probiotic (Table

Table 2 : Microscopic observation of MPKV yeast probiotic

Probiotic yeast isolate	Shape of yeast cell	Size (µm)	Budding habits
I	Oblong	2.85-4.80 × 2.37-2.99	Present
II	Oblong	1.39 × 0.95	Present
III	Elongated	1.13-1.56 × 0.91-0.92	Present
IV	Elongated	1.05-1.15 × 0.70-0.76	Present

Table 3 : Efficiency of NaCl tolerance limit of MPKV probiotics

Probiotic yeast isolate	Growth on NaCl salt (at % concentration)				
	0	5	10	15	20
I	+ +	+ +	++	-	-
II	+ +	+ +	+ +	-	-
III	+ +	+ +	+ +	-	-
IV	+ +	+ +	+ +	-	-

+ + = Full growth of yeast, - = No growth of yeast, I,II,III,IV = Yeast

Table 4 : Growth pattern of MPKV yeast probiotic in different sugar concentration

Incubation period for growth	Probiotic yeast isolate	Growth on sugar concentration (% sucrose)										
		0	5	10	15	20	25	30	35	40	45	50
1 Day	I	+ +	+ +	+ +	+ +	+ +	+	+	+	-	-	-
	II	+ +	+ +	+ +	+ +	+ +	+	+	+	-	-	-
	III	+ +	+ +	+ +	+ +	+ +	+	+	+	-	-	-
	IV	+ +	+ +	+ +	+ +	+ +	+	+	+	-	-	-
2 Days	I	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+	+
	II	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+	+
	III	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+	+
	IV	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+	+
3 Days	I	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
	II	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
	III	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
	IV	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

+ + = Full growth, + = Scanty growth, - = No growth, I,II,III,IV = Yeast

Table 5 : *In vitro* performance of probiotics in the management of post-harvest pathogens of grape

(a) Efficacy of probiotics in dual inoculation (pre spray of post-harvest pathogen followed by post spray of probiotics)

Post harvest pathogens	Pathogen (without probiotics)	Growth of post harvest pathogen in grape fruit bunches sprayed with probiotic			
		Sacro	Sporochek	Darolac	MPKV probiotics
Aspergillus	+	±	±	±	±
Penicillium	+	+	-	-	-
Rhizopus	+	+	±	±	±
Alternaria	+	±	-	-	-

+ = Fungal growth present, ± = Traces of fungal growth, - = No fungal growth.

(b) Efficacy of probiotics on naturally occurring post harvest pathogen in grape bunches (grape bunches sprayed with probiotics)

Pathogens	Growth of Naturally occuring post harvest pathogen in grape bunches sprayed with probiotics				Growth of post harvest pathogens in non probiotic sprayed grape bunches
	Darolac	Sacro	Sporocheck	MPKV Probiotics	
Aspergillus	-	±	±	-	+
Penicillium	-	±	-	-	+
Rhizopus	-	±	-	-	+
Alternaria	-	±	-	-	+

± = Traces of fungal growth, - = No fungal growth.

Table 6 : Field performance of probiotics in the management of post-harvest pathogen of grape

(a) On *Aspergillus* pathogen

Harvesting period after probiotic spray	Presence of *Aspergillus* in grape bunches sprayed with probiotic				
	Darolac	Sacro	Sporocheck	MPKV probiotics	Control (unsprayed)
8 Days	+	+ +	-	-	+
15 Days	+	+	-	-	+

(b) On *Rhizopus* pathogen

Harvesting period after probiotic spray	Presence of *Rhizopus* in grape bunches sprayed with probiotic				
	Darolac	Sacro	Sporocheck	MPKV probiotics	Control (unsprayed)
8 Days	-	+ +	+ +	-	+ +
15 Days	+ +	+ +	+	-	+ +

+ + = Full growth of fungus, + = Scanty growth of fungus, - = No growth of fungus.

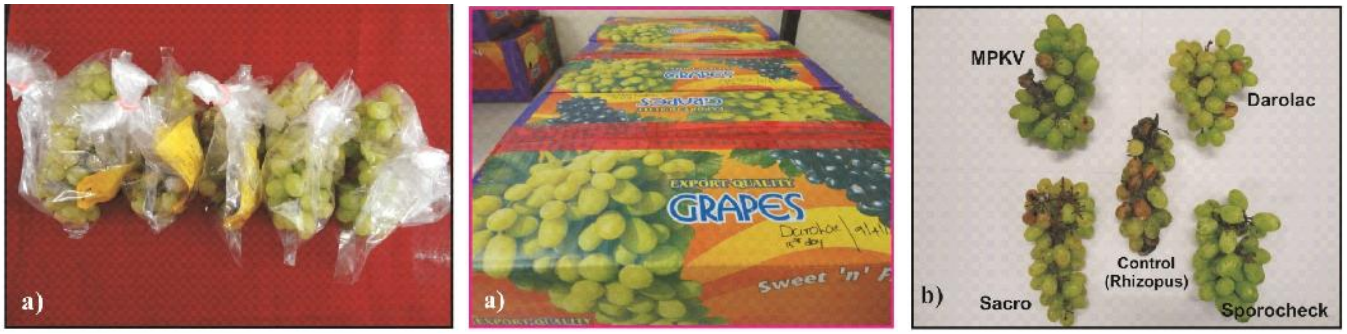

Plate 2 : Performance of Probiotics in management of post harvest diseases on post-harvested grape bunches

6b and Plate 2).

These Probiotics not only used as a biocontrol agent in the management of post harvest diseases and it can also increase the shelf-life of grapes and keep the fruits as fresh as garden harvested (Plate 3).

Kailasapathy and Chin (1999) reported the use of probiotic organisms as live supplements, with particular emphasis on *Lactobacillus acidophilus* and *Bifidobacterium* spp. Probiotic bacteria are increasingly used in food and pharmaceutical applications to balance disturbed intestinal microflora and related dysfunction of the human gastrointestinal tract. The pharmaceutical applications of probiotics has been reported by several other workers also (Molin, 2001; Isolauri *et al.*, 2001;

MPKV probiotics applied grapes

Control grape
(without probiotic application)

Plate 3 : Efects of MPKV Probiotics on grape quality

Shima *et al.*, 2007; Hussein and Aumara, 2006 and Oliveria *et al.*, 2002). However there is no report of probiotic use on plant disease management. This is the first report on use of probiotics in plant disease management.

Conclusion :

Probiotics can be used in the management of post harvest disease and the probiotics can be used to increase the TSS content in the fruits during the cold climate months like January/ February when the sugar conversion in the fruits is not there. The probiotics can also be used to increase the shelf life of the fruits and to keep them as fresh as garden harvested grapes.

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