

EFFECT OF MINERAL COMPONENTS OF THE MEDIUM USED TO GROW BIOCONTROL STRAIN UTPF61 OF *PSEUDOMONAS FLUORESCENS* ON ITS ANTAGONISTIC ACTIVITY AGAINST SCLEROTINIA WILT OF SUNFLOWER AND ITS SURVIVAL DURING AND AFTER THE FORMULATION PROCESS

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SUMMARY

Mineral factors are important in optimizing growth media for production of high quality biocontrol agents. In this work we investigated the effect of some minerals and some combinations of them during fermentation of *Pseudomonas fluorescens* UTPF61 on its growth in flask and fermentor and its subsequent antagonistic activity *in vitro* and *in planta*. Its survival during the formulation process and shelf life over a period of 60 days was investigated. None of the mineral amendments had an effect on bacterial growth. In dual culture assays bacteria grown in a zinc-supplemented medium, showed the maximum inhibition (20 mm) of mycelial growth of *Sclerotinia sclerotiorum*. Medium including a combination of zinc, bromine and iron gave maximum inhibition of mycelial growth. In greenhouse studies, bacteria grown in medium with zinc showed high inhibition against *S. sclerotiorum*. Cobalt, magnesium and copper had a negative effect on antagonistic activity. Bacteria obtained from medium including a combination of zinc and molybdenum showed high antagonistic activity and resulted in 55% healthy plants. A combination of Zn-Fe-Mo improved resistance of the bacterial cells to the drying process. In all treatments (in both flask culture and fermentor studies) UTPF61 survived up to 60 days without any dramatic decline from the initial population.

Key words: mineral factors, formulation, fermentation, sunflower, *Sclerotinia sclerotiorum*.

INTRODUCTION

Sclerotinia wilt, caused by *Sclerotinia sclerotiorum* is one of the most important diseases of sunflower. This soilborne pathogen survives as sclerotia and can infest sunflower seeds, causing damping-off. It also attacks the roots of growing and mature plants, resulting in root

rot, basal stem canker, and wilt (Expert and Digat, 1995). There has been no progress in breeding for resistance to Sclerotinia wilt in sunflower and no effective chemical control of this disease is known (Steadman, 1979).

There is a vast literature describing positive effects of specific strains of rhizobacteria on growth of many plant species in soils in which more or less defined pathogens cause substantial losses (Kloepper *et al.*, 1991). The germination of sclerotia of *S. sclerotiorum*, isolated from carrot discs, was suppressed or reduced by strains of *Bacillus subtilis* (Zazzerini *et al.*, 1987). In a field trial, strains of *Pseudomonas cepacia* increased sunflower emergence in the presence of *S. sclerotiorum* (McLoughlin *et al.*, 1992). In spite of these advantages there is little published on commercial production of these agents.

At present, liquid culture fermentation is the most economical method for producing most microbial biocontrol agents (Jackson, 1997). The production of antibiotics, amino acids, ethanol, and organic acids by submerged culture fermentation has provided an extensive knowledge base for optimizing processes and for designing fermentation vessels for the liquid culture production of biopesticides (Stanbury *et al.*, 1997; Jackson, 1997). In this kind of fermentation, our goal is to obtain a large amount of microbial agent with high efficacy, high resistance to the drying process involved in formulation, and a long shelf life. There are some reports that medium components (carbon and nitrogen sources and their ratios, mineral factors, etc.) and conditions (pH, temperature and oxygen transfer) used to grow biocontrol agents, influence their subsequent properties. Mineral factors are an important part of the medium and need to be optimized. Production of the antibiotic 2,4-diacetylphloroglucinol (DAPG) and its precursor mono-acetylphloroglucinol was stimulated by Zn²⁺ and NH₄Mo²⁺ in liquid culture of *P. fluorescens* Chao (Duffy and Defago, 1999). Production of the siderophore pyochelin in this strain was increased by Co²⁺, and salicylic acid production by NH₄Mo²⁺. Additions of boric acid and sulfates of iron and magnesium enhanced both cell growth and accumulation of the associated antibiotic phenazine 1-carboxylic acid (PCA)

by *P. fluorescens* 2-79 (Slininger and Jackson, 1992). DAPG and PCA have both antibacterial and phytotoxic properties (Gurusiddaiah *et al.*, 1986; Slininger and Shea-Andersh, 2005). These compounds can therefore affect the fermentation of these bacteria and can decrease the germinability of encapsulated seeds (Slininger *et al.*, 1996; Slininger and Shea-Andersh, 2005). Mineral factors may possibly control production of these metabolites during fermentation.

In an industrial fermentation, bacterial inoculants, regardless of their intended use (e.g., agricultural, pharmaceutical, food processing, manufacturing), are inoculated in increasing fermentation volumes, process referred to as scale-up (Smith, 1987). Duffy and Defago (2000) reported the occurrence of spontaneous mutation during the fermentation which could be prevented by addition of zinc and dilution of medium.

In this work, we investigated the effect of adding some minerals, singly or combined, on a) growth of *P. fluorescens* UTPF 61, b) the biocontrol activity of this bacterial strain against *S. sclerotiorum* in *in vitro* and in greenhouse conditions, and 3) its viability during the formulation process. The effect of the most promising combinations of minerals was further studied in the fermentor.

MATERIALS AND METHODS

Biocontrol strain. *P. fluorescens* UTPF 61 was selected from 40 strains of fluorescent pseudomonads on the basis of dual culture assays, growth promotion activity on sunflower and production of HCN and protease (Heidary, 2008). This strain was originally isolated from the rhizosphere of rice. Stock cultures were prepared for storage at -80°C in 1.5 ml vials by mixing equal volumes of 50% glycerol and 24 h culture broth (from single colony inoculum, 25 ml Luria Bertani medium, 100 ml flask, 130 rpm).

Flask culture studies. All assays in this work were performed using a semi-defined medium, containing in grams per litre: yeast extract, 3; sucrose, 10; CaCO₃, 0.4; MgSO₄, 0.4; K₂HPO₄·3H₂O, 0.98. This medium was autoclaved for 15 min at 121°C, and then filter-sterilized. Stock solutions of the following minerals were added separately (micromolar concentrations given in brackets): FeSO₄·7H₂O (36), ZnSO₄·7H₂O (70), H₃BO₃ (19.6), CuSO₄·5H₂O (7), MnCl₂·4H₂O (25), (NH₄)₆Mo₇O₂₄·4H₂O (8.1), CoCl₂·6H₂O (7). In other experiments combinations of minerals were investigated: a) FeSO₄·7H₂O (36) + ZnSO₄·7H₂O (70); b) ZnSO₄·7H₂O (70) + (NH₄)₆Mo₇O₂₄·4H₂O (8.1); c) ZnSO₄·7H₂O (70) + H₃BO₃ (19.6); d) ZnSO₄·7H₂O (70) + FeSO₄·7H₂O (36) + (NH₄)₆Mo₇O₂₄·4H₂O (8.1); e) ZnSO₄·7H₂O (70) + H₃BO₃ + FeSO₄·7H₂O (36); f) ZnSO₄·7H₂O (70) + H₃BO₃ + (NH₄)₆Mo₇O₂₄·4H₂O (8.1);

g) ZnSO₄·7H₂O (70) + H₃BO₃ + (NH₄)₆Mo₇O₂₄·4H₂O (8.1) + FeSO₄·7H₂O (36). All cultures were provided with 134 µM EDTA as a chelating agent. Flask cultures were inoculated using 1 ml of a 24 h NA culture of UTPF 61 adjusted to c. 10⁵ CFU ml⁻¹ in sterile distilled water. Cultures were incubated for 48 h at 130 rpm and 26°C in a rotary shaker incubator, thereafter cell density was measured at 600 nm and cultures were used for formulation, dual culture assays and greenhouse studies. All glassware, including flasks, plates and fermentor vessel used were acid-washed with 0.1% HCl solution to remove residual minerals.

Formulation and air-drying. Medium containing 9×10⁸ CFU ml⁻¹ was used for the preparation of a talc-based formulation. To 400 ml of bacterial suspension, 1 kg of purified talc powder (sterilized at 120°C for 15 h), calcium carbonate 15 g (to adjust the pH to neutral) and carboxymethyl cellulose (CMC) 10 g (adhesive) were mixed under sterile conditions, as described by Vidhyasekaran and Muthuamilan (1995). The product was air dried at 15°C under sterile conditions until moisture content reached 15-20%. After a further 12 h this product was powdered in a grinder for one min and maintained at 4°C in vials. One gram of this formulation was used for viability assays by serial dilution method at determined intervals.

Fermentor studies. A fermentor experiment was designed to mimic the industrial fermentation process in which typically there is stepwise scale-up of batch size (Lam *et al.*, 1994; Schroth *et al.*, 1984). For this purpose 25 ml of King's medium B (King *et al.*, 1954) in a 100 ml flask was inoculated with one ml of bacterial suspension at 10⁵ cfu ml⁻¹ and incubated for 24 h in a rotary shaker at 26°C and 130 rpm. From this culture 500 µl was transferred to 50 ml of fresh medium in a 250 ml flask. After 12 h growth one ml of this medium was used to inoculate 100 ml of fresh medium in a 500 ml Erlenmeyer flask as a seed culture. Finally after 5 h this broth with approximately 10⁶ cells ml⁻¹ in log phase, was transferred to a fermentor under sterile conditions. A 7.5 liter bench-top experimental fermentor (BioFlo 110, New Brunswick Scientific, USA) with a 2 l working volume was operated in batch culture mode. Temperature was controlled at 26°C and the initial pH was adjusted to 7. The pH was monitored by a pH probe (Mettler Toledo 405-DPAS-SC-k85/325, USA) and dissolved oxygen (DO) with a polarographic DO electrode (Ingold Inpro 6800 series USA). The DO probe was calibrated at 0%, (obtained by briefly disconnecting the cable), and at 100%, (obtained using 800 rpm agitation and 5 l min⁻¹ [1 vvm] airflow). An agitation cascade was selected in the controller to maintain DO at 25-30% saturation via automatic adjustment of the agitation speed. To avoid foam formation, castor oil was added in

automatic mode. After the fermentor contents had been sterilized for 25 min at 121°C, filter-sterilized stock solutions of minerals were added to the vessel, and the medium pH adjusted to 7. Cells of *P. fluorescens* UTPF 61 in different stages of growth were harvested in 40 ml aliquots from the fermentor approximately 24 and 48 h after inoculation. Each aliquot was used for formulation, dual culture assays, and greenhouse studies. All fermentor experiments were conducted in triplicate.

Greenhouse studies. To prepare *S. sclerotiorum* inoculum, millet seeds were soaked in water overnight, sterilized (120°C, 20 min, twice), inoculated with mycelial plugs, and incubated at 21°C for three weeks. From this inoculum 0.5 g were mixed with the upper part of sterile soil in plastic pots (diameter, 7.5 cm; depth, 8 cm). For the control treatments, sterile millet seeds were mixed into the soil at the same rate. Sunflower seeds were surface-sterilized with 5% sodium hypochlorite for 3 min and rinsed five times with sterile distilled water. The seeds were then pre-germinated in

15% water agar for 48 h at 26°C in an incubator. Bacterial suspensions were diluted with 1% carboxymethyl-cellulose; germinated seeds were submerged in these suspensions, agitated on a shaker for 45 min and planted 0.5 cm deep in the infested soil. Plants were grown in the greenhouse under day and night temperatures of 25/18°C with a 16 h photoperiod and 75% relative humidity. After 20 days the percentage of healthy plants was determined as described by Expert and Digat (1995).

RESULTS

Effect of mineral factors on growth and antagonistic activity of UTPF61 *in vitro* and *in planta*. In flask culture, none of the mineral amendments (added separately or in combination) had an effect on the growth of bacteria (Tables 1 and 2). In dual culture assays bacteria that were grown in zinc-supplemented medium, showed strong inhibition (20 mm inhibition zone on the medium

Table 1. Antifungal activity of *P. fluorescens* UTPF61 against *Sclerotinia sclerotiorum* grown in media with different mineral amendments. Bacterial cells from 48 h cultures were used for inhibition zone assay on PDA medium.

Mineral amendments	Inhibition zone (mm) ^b	OD ₆₀₀
Control ^a	14.66 b	2.10a
MnCl ₂	17.66 b	2.13 a
CoCl	16.00 b	2.17 a
H ₃ BO ₃	17.50 ab	2.09 a
FeSO ₄	20.00 ab	2.13 a
ZnSO ₄	17.50 a	2.10 a
CuSO ₄	16.00 ab	2.05 a
(NH ₄) ₆ Mo ₇ O ₂₄	17.33 ab	2.06 a

^aMedium without mineral amendments.

^bWidth of the zone of inhibition was measured from the edge of the fungal colony to the edge of the bacterial colony. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test.

Table 2. Antifungal activity of *P. fluorescens* UTPF61 against *Sclerotinia sclerotiorum* grown in media with different combinations of mineral amendments. Bacterial cells from 48 h cultures were used for inhibition zone assay on PDA medium.

Mineral amendments	Inhibition zone (mm) ^b	OD ₆₀₀
ZnSO ₄ + H ₃ BO ₃ + FeSO ₄	17.25 a	2.205 a
ZnSO ₄ +(NH ₄) ₆ Mo ₇ O ₂₄ + FeSO ₄	9.5 cd	2.205 a
ZnSO ₄ +(NH ₄) ₆ Mo ₇ O ₂₄ + FeSO ₄ + H ₃ BO ₃	10.5 cd	2.190 a
ZnSO ₄ + FeSO ₄	13.75 b	2.207 a
Control ^a	12.25 cb	2.180 a
ZnSO ₄ + H ₃ BO ₃	8.5 d	2.209 a
ZnSO ₄ +(NH ₄) ₆ Mo ₇ O ₂₄	8.5 d	2.216 a
ZnSO ₄ +(NH ₄) ₆ Mo ₇ O ₂₄ + H ₃ BO ₃	7.5 d	2.200 a

^aMedium without mineral amendments.

^bWidth of the zone of inhibition was measured from the edge of the fungal colony to the edge of the bacterial colony. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test.

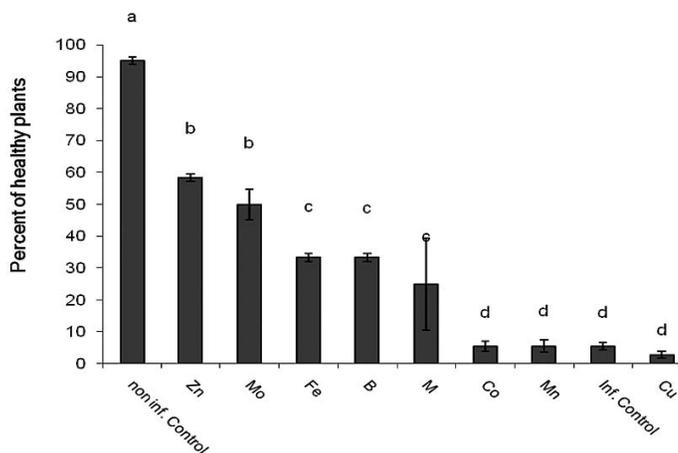


Fig. 1. Biocontrol activity of *Pseudomonas fluorescens* UTPF61 grown in medium with different mineral amendments, against *Sclerotinia sclerotiorum* under greenhouse conditions. M: medium without mineral amendments. Percent of healthy plants determined after 20 days. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test.

used) against *S. sclerotiorum*, and also those cells from medium with the combination zinc, bromine and iron showed high inhibition (17.2 mm). In some combinations inhibitory activity of bacteria decreased (Table 2).

In greenhouse studies, bacteria grown in medium containing zinc showed strong inhibition of *S. sclerotiorum*, yielding 58.3% healthy plants after 20 days and in the case of molybdenum 49.9%. Cobalt, magnesium and copper had a negative effect on antagonistic activity (Fig. 1). Bacteria obtained from medium with a combination of zinc and molybdenum were highly inhibitory, with 55.5% healthy plants in this case (Fig. 2). In con-

trast, the combinations Zn-Mo-Fe and Zn-Fe reduced the antagonistic activity of the bacterium (Fig. 2).

The population of UTPF61 decreased about 2.61-4.29 \log_{10} CFU g^{-1} in talc-based formulations during the formulation process except in the case of Zn-Fe-Mo where its decline was only 0.85 \log_{10} CFU g^{-1} in dry weight of air dried product (Fig. 3). In all treatments UTPF61 survived up to 60 days without any dramatic decline from the initial population (Fig. 3). Zn-B and Co increased the sensitivity of cells to air drying, and cell populations were decreased about 4.04-4.39 \log_{10} CFU g^{-1} in the talc-based formulation.

As was found in the flask culture studies, the different mineral treatments had no effect on growth of the bacteria in the fermentor (Table 3). In three of the fermentation processes, cells from a 24 h culture (early stationary phase) showed larger inhibition zones than those from 48 h. The antagonistic activity of bacteria drastically decreased after 48 h (late stationary phase) in the control batches.

In greenhouse studies, Zn and Zn-Mo combined improved antagonistic activity of UTPF61 against the pathogen when using 24 h as well as 48 h cultures (Fig. 4). In all treatments there were no significant differences in antagonistic activity between 24 h and 48 h cultures.

Tolerance of cells to air drying was similar in all treatments (Fig. 5) and there was no significant decrease in viability after the population stabilized over a period of 60 days at 4°C.

DISCUSSION

The medium used in this study was a semi-defined medium with 3 $g l^{-1}$ yeast extract. This medium is simi-

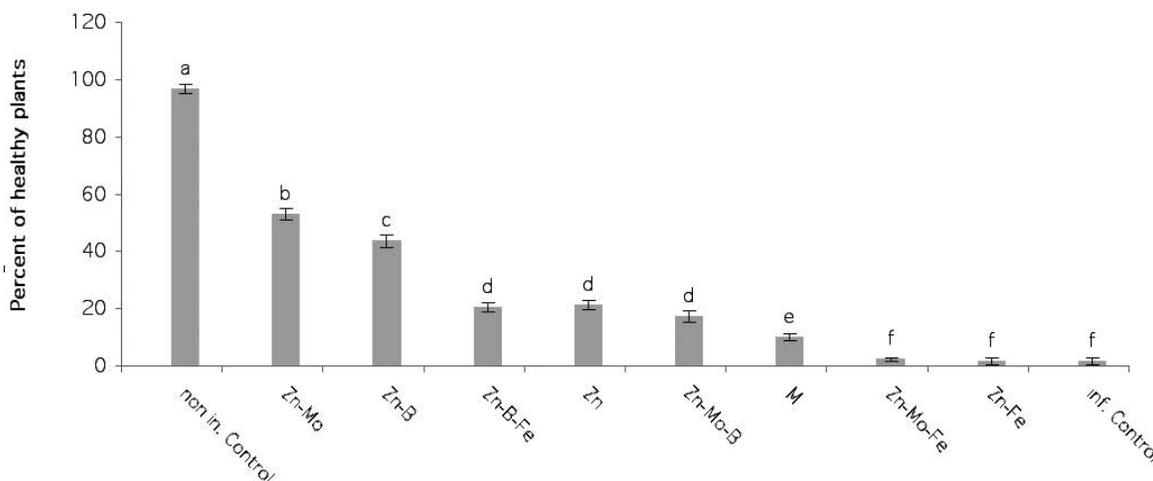


Fig. 2. Biocontrol activity of *Pseudomonas fluorescens* UTPF61 grown in medium with different combinations of mineral amendments, against *Sclerotinia sclerotiorum* under greenhouse conditions. Percent of healthy plants determined after 20 days. M: medium without mineral amendments. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test.

Table 3. Antifungal activity of *P. fluorescens* UTPF61 against *Sclerotinia sclerotiorum* grown in fermentor with different mineral amendments. Bacterial cells at different physiological stages were used for inhibition zone assay on PDA medium.

Mineral amendments at different physiological states	Inhibition zone (mm) ^b	OD ₆₀₀
ZnSO ₄ at 24 h	23.3 a	2.71 a
ZnSO ₄ at 48 h	21.6 ab	2.78 a
ZnSO ₄ +(NH ₄) ₆ Mo ₇ O ₂₄ at 24h	20.6 ab	2.78 a
Control ^a at 24 h	20.6 ab	2.207 a
ZnSO ₄ +(NH ₄) ₆ Mo ₇ O ₂₄ at 48 h	17.3 b	2.180 a
Control at 48 h	0.3 c	2.209 a

^aMedium without mineral amendments.

^bWidth of the zone of inhibition was measured from the edge of the fungal colony to the edge of the bacterial colony.

Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test.

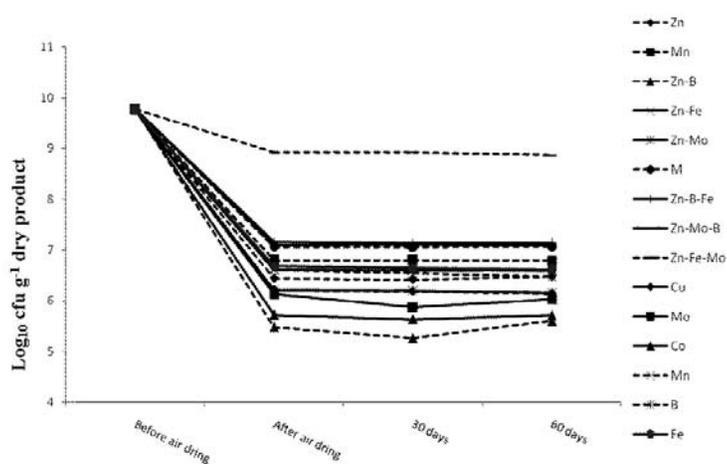


Fig. 3. Survival of *Pseudomonas fluorescens* UTPF61 grown in medium with different mineral amendments during air drying and maintenance of its talc-based formulation for 60 days in vials at 4°C.

lar to that used in commercial fermentations. In our fermentations yeast extract was used as sole nitrogen source and alternative carbon source. This nutrient provides some minerals and vitamins, too. Additions of more mineral factors and different combinations of it to this medium had no effect on growth of UTPF61 in this study, as opposed to their effect on antagonistic activity of this strain. These observations are in agreement with those reviewed by Weinberg (1970) about nine trace elements of biological interest (Atomic numbers 23-30, 42). Of these nine, the concentrations of manganese, iron and zinc are the most critical in secondary metabolism. In every secondary metabolic system in which sufficient data have been reported, the yield of the product varies linearly with the logarithmic concentration of the 'key' metal. The linear relationship does not apply at concentrations of the metal which are either insufficient, or toxic, to cell growth.

There are reports that medium and conditions used

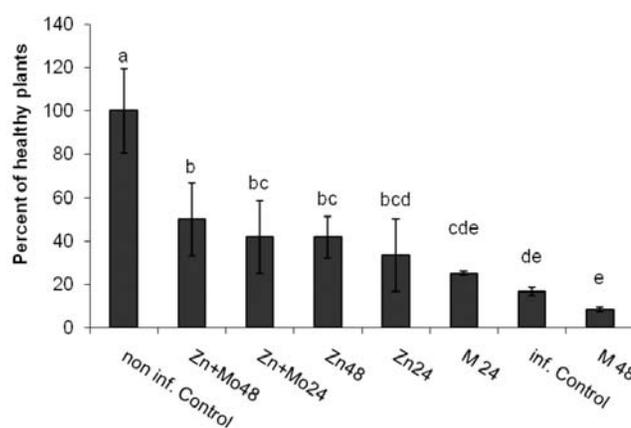


Fig. 4. Biocontrol activity of *Pseudomonas fluorescens* UTPF61 against *Sclerotinia sclerotiorum* grown in a fermentor with different mineral amendments under greenhouse conditions. M: medium without mineral amendments. Percent of healthy plants determined after 20 days. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test.

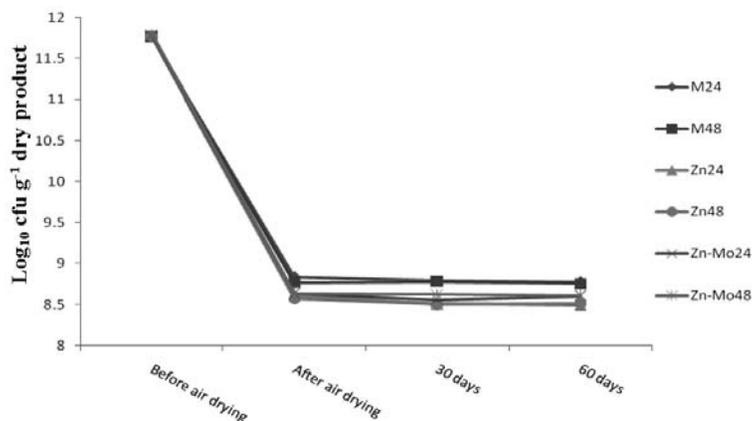


Fig. 5. Survival of *Pseudomonas fluorescens* UTPF61 grown in a fermentor with different mineral amendments during air drying and maintenance of its talc-based formulation for 60 days in vials at 4°C.

for production of biocontrol agents, influence their ability to survive during the formulation process (Slininger *et al.*, 1996; Zhang *et al.*, 2005). For example mild thermal, and pH stresses and carbon starvation can increase the resistance of cells to further stresses (van Overbeek *et al.*, 1995; Givskov *et al.*, 1994a, 1994b; Jenkins *et al.*, 1988; Oestling *et al.*, 1993). There are different hypotheses to explain these phenomena. In most of these studies resistance of cells was the result of expression of shock-inducible proteins like heat and cold shock proteins. In our study bacterial populations that were grown in medium with a combination of molybdenum, zinc and bromine decreased only 0.85 log₁₀ CFU g⁻¹ dry weight of air-dried product after formulation, whereas it was at least 2.4 log₁₀ in other formulations. Perhaps this combination of minerals stimulated expression of stress-tolerance proteins.

In most of the treatments zinc solely or in combination with molybdenum improved the biocontrol activity of UTPF61 after fermentation. There are some reports that zinc improves production of the antibiotics phenazine (Ownley *et al.*, 2003; Slininger and Jackson, 1992) and 2,4-diacetylphloroglucinol (Duffy and Défago, 1997, 1999). Perhaps zinc amendments to cells in our cultures altered their gene expression towards higher antibiotics production after introduction to the rhizosphere. Production of large amounts of antibiotics during fermentation could, however, have a negative effect on growth, and if the end product of formulation is encapsulated on seeds, it could negatively influence germination.

In fermentor studies we used a stepwise procedure for production of inoculum as a mimic of industrial fermentation, a process referred to as scale-up (Smith, 1987). Antagonistic activity of cells from this quasi-industrial process that were subjected to zinc amendments, was significantly better than that of other treatments without this mineral.

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