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***In vitro*, *in vivo* and *in situ* Evaluation of
Fungicides Tested Individually or in Combination for the
Control of the *Fusarium* Dry Rot of Potato**

¹M. Daami-Remadi, ²F. Ayed, ²H. Jabnoun-Khiareddine,
²K. Hibar and ²M. El Mahjoub

¹National Institute of Agronomic Research of Tunisia,
PRRDA-CE 4042 Chott-Mariem, 4042, Sousse, Tunisia

²High School of Horticulture and Breeding of Chott-Mariem, 4042, Sousse, Tunisia

Abstract: Several fungicides are tested individually or in dual combination against four *Fusarium* species causing potato tuber dry rot in Tunisia. Incorporated into the culture media PDA, the tested fungicides significantly inhibited the mycelial growth of all *Fusarium* species incubated at 25°C for 6 days; a significant interaction was observed between the both fixed factors ($p \leq 0.05$). Applied on potato tubers (tuber immersion for 10 min) prior inoculation, all tested fungicides, combined or not, have significantly reduced by more than 50%, comparatively to the untreated controls, the development of dry rot occasioned by *Fusarium* sp. after 21 days of incubation at 25-27°C. A significant interaction was observed between the treatments and the *Fusarium* species ($p \leq 0.05$). In natural conditions, tuber treatment by the tested fungicides, prior their definitive storage, has reduced dry rot development by about 50%. A synergistic effect was observed *in vitro*, *in vivo* and *in situ* between the mixed fungicides traduced by a better efficacy, in comparison to their individual effects, showing their compatibility and the promotion of the disease control. These combined fungicides could play a role in an integrated pest management against potato tuber-borne pathogens.

Key words: *Solanum tuberosum* L, interaction, chemical control, *Fusarium* complex, synergism

Introduction

In Tunisia, potato tuber dry rot is caused by a complex of *Fusarium* species which incidence and frequency are depending on production zones. *F. solani*, *F. oxysporum* and at a lesser frequency *F. sambucinum* and *F. graminearum* are the most isolated from local tubers showing dry rot symptoms (Daami-Remadi and El Mahjoub, 1996, 2004, 2006; Daami-Remadi *et al.*, 2006; Priou and El Mahjoub, 1999; Chérif *et al.*, 2001).

Benzimidazoles and conazoles fungicides were used since 1970 (Leach, 1971; Murdock and Wopd, 1972; Tisdale and Lord, 1973). Benzimidazoles such as benomyl, carbendazim, thiophanate-methyl and thiabendazole are used against *Fusarium* and *Phoma exigua*, *Helminthosporium solani*, *Rhizoctonia solani* (Leach and Nielsen, 1975; Tivoli *et al.*, 1986; Carnegie *et al.*, 1990, 1998; Bang, 1992; Kawchuck *et al.*, 1994; Mérida and Loria, 1994; Errampalli and Johnston, 2001).

Interaction of local *Fusarium* population with benzimidazoles and prochloraz depends on *Fusarium* species and tested doses used *in vitro*. Thiophanate-methyl and prochloraz inhibited dry

rot development on tubers inoculated by *F. sambucinum*, (Daami-Remadi and El Mahjoub, 1996, 1997). Chérif *et al.* (2001) showed efficacy of thiophanate-methyl and carbendazim on germination, mycelial growth and sporulation of a local *F. roseum* var. *sambucinum* isolate.

In Tunisia, several rots affect potato tubers in the field and in unrefrigerated rustic stores consisting of heaping tubers in a shaded and aerated place and covering them with a thick layer of straw, weeds or potato haulms. Tuber losses noted during this traditional storage, are mainly due to dry rot, leak caused by *Pythium aphanidermatum* and *P. ultimum* and pink rot occasioned by *Phytophthora erythroseptica* that threat a strategic crop in Tunisia (Daami-Remadi and El Mahjoub, 1996; Triki *et al.*, 1996, 2001; Triki and Priou, 1997, Priou and El Mahjoub, 1999; Chérif *et al.*, 2001; Daami-Remadi, 2001 a,b).

Chemical control of local *Pythium* sp. and *Phytophthora* sp. isolates was achieved by several fungicides tested *in vitro* and *in vivo* such as hymexazol and metalaxyl-mancozeb (Triki and Priou, 1997, Triki *et al.*, 2001, Daami-Remadi, 2001a). In natural conditions, tubers often showed mixed infections of causal agent rot including leak, dry and pink rots. Furthermore, *in vitro* screening for local *Fusarium* sp. resistance to some benzimidazoles showed that *F. solani*, *F. oxysporum* f. sp. *tuberosi* and *F. graminearum* isolates are susceptible to these fungicides whereas *F. sambucinum* isolates are resistant (Daami-Remadi and El Mahjoub, 2006a). These chemicals with a single-site mode of action such as benzimidazoles are more likely to lead to development of resistance (Kawchuck *et al.*, 2002). Programs incorporating fungicides with different modes of action to optimise disease control and minimize the risk of resistance development are used in resistance prevention strategies (Beresford, 1994). Consequently, for reducing tuber losses in storage, an integrated control strategy should be followed against all potato post-harvest pathogens and application of mixtures of fungicides may help to slow down the development of resistance. The main purpose of the present study focused on the evaluation of efficacy of several fungicides, with different target effects against *Fusarium* or Pythiaceae, tested individually or in combination for the chemical control of the causal agents of potato tuber dry rot *in vitro*, *in vivo* and *in situ*.

Materials and Methods

Pathogens

F. solani, *F. graminearum*, *F. sambucinum* and *F. oxysporum* f. sp. *tuberosi* are isolated on 2002 from tubers of cv. Spunta showing typical symptoms of dry rot. *Fusarium* sp. are grown at 25°C on PDA for one week. They are stored at -20°C in 20% glycerol solution for long term preservation.

Potato Cultivars

Tubers cv. Spunta, the most cultivated in Tunisia, are used in this current study. They are obtained, on 2003, from the Technical Centre of Potato of Tunisia. For laboratory experiments, tubers are stored in the darkness at 6°C and brought to room temperature three hours before use. In *in situ* experiments, tubers are stored traditionally.

Fungicides

Tested fungicides are subscribed in the Tunisian phytosanitary index (Anonymous, 2003) and which active ingredient components are showed to be efficient against Pythiaceae and/or *Fusarium* sp.. Main tested fungicides characteristics are presented in Table 1.

In vitro Activity of Tested Fungicides Against *Fusarium* sp.

Fungicides are dissolved in sterile distilled water before their incorporation (1%v/v), following chosen doses (Table 1), in PDA in surfusion. A culture media added with a same quantity of ethanol serves as untreated control (dose 0 mg L⁻¹). After solidification, agar discs (of 6 mm in diameter) colonized by the pathogen are placed in the center of the petri dish.

Table 1: Characteristics and doses of fungicides tested against *Fusarium* spp. causing potato tuber dry rot

Active ingredients (a.i)	Trade names (tn)	Concentrations of a.i	Tested doses (tn)
Benomyl (BE)	Benlate 50	50%	60 g.hL ⁻¹
Carbendazim (BA)	Bavistine	50%	50 g.hL ⁻¹
Cymoxanil+Mancozeb (FU)	Fulvax 2000	6%+70%	170 g.hL ⁻¹
Chlorothalonil+Propamocarb (TC)	Tatto-C	375 g L ⁻¹ +375 g L ⁻¹	200 mL.hL ⁻¹
Thiabendazole+Flutriafol (VN)	Vincit F	25 g L ⁻¹ +25 g L ⁻¹	300 mL.hL ⁻¹
Fludioxonil+Cyprodinil (SW)	Switch Wp	25%+37.5%	200 g.hL ⁻¹
Metalaxyl+Mancozeb (R)	Ridomil MZ 58	10%+48%	200 g.hL ⁻¹

Inhibitory activity of fungicides is evaluated on mycelial growth of tested *Fusarium* sp. estimated via mean colony diameter formed after 6 days of incubation at 25°C. Tested fungicides are applied in culture media individually or in dual combination searching for compatibility between fungicides and synergistic activity against *Fusarium* sp.

Statistical analysis (ANOVA) are performed following a completely randomised factorial design where treatments (fungicides and untreated control) and *Fusarium* sp. are both fixed factors. Means are separated using Fisher's protected LSD test ($p \leq 0.05$).

In vivo Activity of Tested Fungicides Against *Fusarium* sp.

Efficacy of fungicides previously tested *in vitro* was estimated via development of dry rot on inoculated and treated tubers.

Tubers (cv. Spunta) are superficially disinfected with a solution of 10% sodium hypochlorite, for 5 min and then rinsed abundantly with sterile distilled water. Container and alveolus plaques used for inoculated tubers incubation, are washed before use, dipped for 24 h in sodium hypochlorite solution then rinsed with sterile distilled water.

Fungicides tested individually or in combination, are suspended in water according to tested doses and tuber treatment was realized by dipping tubers, during 10 min, in a fungicidal suspension prior inoculation. Inoculation technique consists of depositing an agar disc (6 mm diameter) colonized by pathogen at occasioned wounds (6 mm diameter and depth). Tuber incubation is realized at 25-27°C for 21 days at high relative humidity. Every elementary treatment is repeated twenty times (ten tubers×two wounds).

After incubation period, tubers were cut longitudinally via sites of inoculation. Parameters of dry rot induced (maximal width (w) and depth (d)) are noted. The pathogen penetration within tubers is calculated following formula of Lapwood *et al.* (1984) where:

$$\text{Penetration (mm)} = (w/2 + (d - 6))/2$$

Statistical analysis (ANOVA) are performed following a completely randomised factorial design where treatments (fungicides and untreated control) and *Fusarium* sp. are both fixed factors. Means are separated using Fisher's protected LSD test ($p \leq 0.05$).

In situ Activity of Tested Fungicides Against *Fusarium* sp.

Fungicides, tested *in vitro* and *in vivo* are also assessed for their *in situ* efficacy against dry rot development on treated tubers in comparison to controls. One month after harvest, tubers are washed with tap water and then treated by immersion during 10 min into fungicide suspension prepared according to doses presented in Table 1. Untreated control tubers are dipped in water. Treated tubers are then air dried in boxes prior their storage. Elementary treatments are placed on new straw which is also used for their separation and covering. Each elementary treatment consists of 50 kg of tubers and is repeated three times.

Tuber inspection is realized every 15 days during a storage period of two months. Every elementary treatment is observed, tuber by tuber and rotten tubers are counted then eliminated for avoiding contamination of the remaining healthy ones. At the end of the essay, the total rotten tuber number, per every elementary treatment, is calculated.

Statistical analysis (ANOVA) are performed following a completely randomised design where treatments (fungicides and untreated control) represent the fixed factor and the number of rotten tubers is the dependant variable. Means are separated using Fisher's protected LSD test ($p \leq 0.05$).

Results

Effects of Fungicides on Mycelial Growth of Fusarium sp.

The effect of some fungicides, incorporated in the culture media individually or in dual combination, are tested for *in vitro* development of *Fusarium* sp. Table 2 showed mycelial growth of the tested *Fusarium* sp. obtained at realized treatments.

Mean colony diameter, formed after 6 days of incubation at 25°C, varied upon tested *Fusarium* species and treatments revealing existence of a significant interaction (at $p \leq 0.05$) between both fixed factors.

All fungicides applied *in vitro*, individually or in combination, significantly reduced mycelial growth of all tested *Fusarium* species in comparison to untreated controls. More than 90% of mycelial growth inhibition was reached in *F. graminearum*, *F. oxysporum* and *F. solani* for treatments BE, BA, R+BE, R+BA, TC+SW, FU+SW and FU+VN. However, the interaction between *F. sambucinum* and the other tested fungicides was different. In fact, for benzimidazoles fungicides such as benomyl (BE) and carbendazim (BA), pathogen growth was reduced by only 26 to 46%, comparatively to untreated control, in comparison to 90% noted for the other three *Fusarium* species.

These both fungicides showed a synergistic effect with the treatment R (Metalaxyl+mancozeb) where 85 to 90% of *F. sambucinum* inhibition was obtained.

The treatment VN (thiabendazole+flutriafol) totally inhibited mycelial growth of *F. oxysporum* and *F. solani* and at a lesser degree *F. sambucinum* and *F. graminearum*. Table 2 revealed, *in vitro* synergistic effect of the entire combinations of tested fungicides (R+BE, R+BA, TC+SW, TC+VN, FU+SW, FU+VN) against all *Fusarium* species; an important inhibitory effect was obtained in comparison to fungicides individually applied.

Effects of Fungicides on Fusarium sp. Aggressivity on Tubers

Table 3 showed mean pathogen penetration in tubers cv. Spunta, individually inoculated by *Fusarium* species, noted after 21 days of incubation at 25-27°C, depending on different treatments. Obtained results revealed existence of a significant interaction (at $p \leq 0.05$) between treatments and *Fusarium* sp. All tested fungicides applied individually or in dual combination have significantly limited dry rot development on inoculated tubers by more than 50%. Maximum inhibition was reached with the treatments R+BA, TC+SW where development of dry rot regressed by more than 90%, in comparison to inoculated and untreated control in the case of *F. graminearum*.

Synergistic effects of combined fungicides were also obtained *in vivo*. In fact, mean penetration, noted for each combined treatment, regressed by more than 50% for all treatments. Inhibition reached 90% for mixed fungicides, in comparison to inoculated untreated control and individually treatments. This is the case of combined treatments R+BA, TC+SW, FU+SW, FU+VN and at a lesser degree, R+BE and TC+VN where dry rot inhibition was estimated by 74 and 82% respectively, in comparison to inoculated untreated control.

Table 2: Effect of some fungicides incorporated into culture media PDA, individually or in dual combination, on mycelial growth of four *Fusarium* species as measured by the mean colony diameter (cm) noted after 6 days of incubation at 25°C

Treatments/Pathogens	<i>F. gram.</i>	<i>F. samb.</i>	<i>F. solani</i>	<i>F. oxysp.</i>
Control	7.90	7.47	7.90	7.85
R	5.95	2.70	3.87	5.02
BE	0.55	5.52	0.00	0.57
BA	0.70	4.00	0.00	0.37
R+BE	0.00	1.07	0.00	0.45
R+BA	0.00	0.67	0.15	0.00
TC	4.62	3.92	1.97	4.25
FU	0.92	0.37	2.02	3.07
SW	0.97	1.22	4.80	3.07
VN	3.07	2.00	0.00	0.00
TC+SW	0.25	0.70	0.70	2.42
TC+VN	2.47	1.72	0.95	0.00
FU+SW	0.00	0.00	0.27	1.07
FU+VN	0.15	0.25	0.00	0.00

R: metalaxyl-mancozeb, BE: benomyl, BA: carbendazim, TC: chlorothalonil-propamocarb, FU: cymoxanil-mancozeb, SW: fludioxonil-cyprodinil, VN: thiabendazole-flutriafol, *F.gram.*: *F. graminearum*, *F. samb.*: *F. sambucinum*, *F. oxysp.*: *F. oxysporum* f. sp. *tuberosi*, LSD (Treatments x *Fusarium* sp.) = 0.18 cm ($p \leq 0.05$)

Table 3: Effect of some fungicides applied, individually or in dual combination, on dry rot development occasioned by *Fusarium* species as measured by the mean pathogen penetration (mm) into inoculated tubers noted after 21 days of incubation at 25-27°C

Treatments/Pathogens	<i>F. gram.</i>	<i>F. samb.</i>	<i>F. solani</i>	<i>F. oxysp.</i>
Control	16.10	18.75	7.52	7.77
R	10.67	13.45	5.40	5.52
BE	9.92	5.80	4.05	4.17
BA	2.77	11.60	5.35	5.42
R+BE	4.07	6.32	3.40	3.40
R+BA	1.50	10.52	3.05	3.05
TC	4.07	10.75	6.22	6.20
FU	6.92	7.50	6.30	6.37
SW	7.52	3.57	3.70	3.72
VN	6.27	9.37	3.15	3.17
TC+SW	1.50	3.12	3.45	3.52
TC+VN	3.02	6.77	3.05	3.17
FU+SW	1.50	2.65	3.37	3.55
FU+VN	1.72	9.50	2.50	2.55

R: metalaxyl-mancozeb, BE: benomyl, BA: carbendazim, TC: chlorothalonil-propamocarb, FU: cymoxanil-mancozeb, SW: fludioxonil-cyprodinil, VN: thiabendazole-flutriafol, *F.gram.*: *F. graminearum*, *F. samb.*: *F. sambucinum*, *F. oxysp.*: *F. oxysporum* f. sp. *tuberosi*, LSD (Treatments x *Fusarium* sp.) = 2.8 mm ($p \leq 0.05$)

Table 4: Effect of tuber (cv. Spunta) treatment by fungicides, applied individually or in combination on dry rot development observed after two months of a traditional storage

Treatments	Mean number of rotten tubers noted on a elementary lot of 150 kg
Control	25.6
R	16.6
BE	16.6
BA	16.6
R+BE	16.3
R+BA	16.6
TC	17.6
FU	24.0
SW	21.0
VN	16.6
TC+SW	19.0
TC+VN	17.0
FU+SW	15.3
FU+VN	14.3

R: metalaxyl-mancozeb, BE: benomyl, BA: carbendazim, TC: chlorothalonil-propamocarb, FU: cymoxanil-mancozeb, SW: fludioxonil-cyprodinil, VN: thiabendazole-flutriafol

Effects of Fungicides on Dry Rot Development in Natural Conditions of a Traditional Storage

The effect of fungicides, applied individually or in dual combination, was evaluated on potato tubers cv. Spunta, not previously inoculated.

Analysis of variance revealed that the number of rotten tubers, observed on different lots of 150 kg after two months of a traditional storage, did not significantly vary upon applied treatments. However, Table 4 showed that most tested tuber treatments, applied prior final storage, inhibited development of dry rot in store by more than 50% comparatively to untreated controls.

Synergistic effects are observed for different fungicide combinations and dry rot development during traditional storage regressed by 45 to 59%, in comparison untreated control.

Discussion

Potato tuber dry rot is a post harvest disease with an increasingly importance in Tunisia. The complex of *Fusarium* species involved in disease development, its aggressivity on tubers and potato plants also as wilting agents (Daami-Remadi and El Mahjoub, 2004) and its survival in fields justified necessity of a tuber treatment in addition to respect of prophylactic methods.

The majority of benzimidazoles fungicides, tested in the current study against *Fusarium* sp. (responsible of tuber dry rot in Tunisia) showed an interaction with the fusarial complex. In fact, *F. oxysporum* f. sp. *tuberosi*, *F. solani* and *F. graminearum* are susceptible and are inhibited by more than 90% whereas *F. sambucinum* seems to react differently. Our results dealing with efficacy of benzimidazoles against development of local *Fusarium* sp. join other findings (Leach and Nielsen, 1975; Carnegie and Cameron, 1992; Kawchuk *et al.*, 1994; Theron and Millard, 1996; Daami-Remadi and El Mahjoub, 1997; Chérif *et al.*, 2001; Ali *et al.*, 2005). The present study showed interspecific difference within the tunisian potato *Fusarium* complex as estimated by their susceptibility to these fungicides. This result joins our previous findings in a comparative study of local *F. solani* var. *coeruleum* isolates and *F. sambucinum*, *F. graminearum* and *F. culmorum* isolated from imported rotten tubers (Daami-Remadi and El Mahjoub, 1997). This Fungicides x *Fusarium* sp. interaction was also observed by Carnegie *et al.* (1998) who found that, in the case of *Fusarium solani* var. *coeruleum*, fenpiclonil and combined thiabendazole-imazalil are more active than imazalil alone.

Chérif *et al.* (2001) found that iprodione, metalaxyl-mancozeb and procymidone were efficient against development of one tested *F. sambucinum* isolate. Metalaxyl-mancozeb, tested against our *Fusarium* sp. isolates, significantly reduced their mycelial growth comparatively to untreated controls. Its interaction varied from 25% for *F. graminearum* to 64% for *F. sambucinum*; an intermediate result was obtained in the case of *F. oxysporum* f. sp. *tuberosi* and *F. solani*. Its activity against *Fusarium* sp. is mainly due to mancozeb because metalaxyl is known to be active against Oomycota fungi and it is used against Pythiaceae infecting potato such as *Pythium* sp. and *Phytophthora* sp. (Inglis *et al.*, 1999; Powelson and Inglis, 1999; Taylor *et al.*, 2002, 2004; Peters *et al.*, 2001, 2003; Wong and Wilcox, 2001). This same fungicide composed of metalaxyl-mancozeb also showed inhibitory activity against tunisian isolates of *Pythium* sp. and *Phytophthora erythroseptica* (Triki and Priou, 1997; Daami-Remadi, 2001b). Large activity of metalaxyl-mancozeb constitutes an alternative for the control of the entire fungal complex of tubers in storage.

This present study showed that several combined treatments such as R+BE (metalaxyl-mancozebe+benomyl), R+BA (metalaxyl-mancozebe+carbendazim), TC+SW (Chlorothalonil-Propamocarbe+Fludioxonil-Cyprodinil), TC+VN (Chlorothalonil-Propamocarbe+Thiabendazole-Flutriafol), FU+SW (Cymoxanil-Mancozeb+Fludioxonil-Cyprodinil), FU+VN (Cymoxanil-Mancozeb+Thiabendazole-Flutriafol) inhibited the mycelial growth of all tested *Fusarium* species including *F. sambucinum* which was found to be resistant to three tested benzimidazoles (Daami-Remadi and El Mahjoub, 2006a). The inhibition obtained with mixed fungicides is more important than

that achieved with fungicides tested individually. This finding showed presence of a synergistic effect between them, when combined, without affecting their inhibitory activity. These same combined treatments showed efficacy against *in vitro* development of *Pythium* sp. and *Phytophthora erythroseptica* (unpublished data). Powelson and Inglis (1999) showed that dimethomorph+mancozeb, cymoxanil+mancozeb and propamocarb hydrochloride+chlorothalonil, when applied to the seed piece prior inoculation with *Phytophthora infestans*, significantly increased sprout emergence compared to the inoculated water control.

The majority of fungicide combinations also limited *in vivo* development of dry rot on artificially inoculated tubers in laboratory experiments and also in natural conditions of a traditional storage. These different combinations could play a role in an integrated control strategy against all tuber fungal agents. Furthermore, although *in situ* experiments are realized relatively late, about one month after harvest, results noted even at this late application are acceptable. In fact, used tubers are sorted out and washed prior treatment, the fungicide suspensions are renewed after several tuber dipping and the choice of an aired local for tuber storage are the main components participating in optimisation of efficacy of tested fungicide treatments.

Finally, as in Tunisia all potato cultivars are susceptible with variable degrees to *Fusarium* sp. (Daami-Remadi and El Mahjoub, 1996; Ayed *et al.*, 2006a) and the cultivar Spunta, the almost exclusively used, is also susceptible to the potato post-harvest pathogens (Priou *et al.*, 1997). Furthermore, major traditionally stored tubers are used for late autumn culture, treatment prior storage is indispensable for avoiding emergence and wilt problems. Certain tested fungicides also have an effect in promoting plant development in the field when seeds are treated with fungicides prior plantation (Ayed *et al.*, 2006b).

The present study proposed different individually or combined treatments, tested in their majority for the first time in Tunisia, for chemical control of *Fusarium* dry rot, reducing incidence of this post-harvest disease and optimising its control. A large range of fungicides, belonging to different chemical families are tested and synergistic effects are obtained with combined treatments *in vitro*, *in vivo* and *in situ*. These chemicals could play an important role in tuber protection in stores and could avoid soil infestation when seeds are previously treated.

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