Czech J. Genet. Plant Breed., 38, 2002 (2): 82–86

SHORT COMMUNICATION

The Effects of Silver on Microbial Contamination of Agar Medium and on Interactions between Mildew and Barley Leaf Segments with and without the *mlo* Gene

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Abstract: Segments of primary leaves of several barley varieties with and without the *mlo* gene were placed in Petri dishes on an agar medium containing benzimidazole, mineral nutrients and 0, 0.1, 0.3, 1 and 3 ppm of $AgNO_3$. Three Petri dishes were prepared of each concentration. The segments were uniformly inoculated with 10^3 conidia/cm^2 of the partially *mlo*-virulent powdery mildew culture PV-32. Subsequently, one open Petri dish of each Ag-concentration was exposed for 1 hour to a different potential contamination environment: one in the laboratory (low load), one in a humid cellar close to stored vegetables (medium load) and one on the top of a compost heap of decaying garbage (heavy load). Germination of mildew spores on the medium surface declined slightly with increasing concentration of $AgNO_3$. Mildew infection was evaluated 7 days after inoculation. The number of mildew colonies per leaf segment and the differential interaction of the *Mlo*- and *mlo*-varieties with the mildew culture was apparently not affected by the $AgNO_3$ concentration. Contamination of the medium by airborne micro-organisms was evaluated 12 days after exposure both microscopically and by eye. The contamination of the medium increased with environmental load and with decreasing $AgNO_3$ concentration. 0.1 ppm $AgNO_3$ markedly retarded the growth of contaminant colonies from all three environments, but did not prevent contamination. At 1 ppm $AgNO_3$, no contamination was observed on the media exposed to low and medium load, but several dozen small contaminant colonies developed on the medium exposed to heavy load. At 3 ppm $AgNO_3$, only three small contaminant colonies developed on the medium exposed to heavy load, while the media exposed to medium and low load remained clean. It can be concluded that adding 1 ppm $AgNO_3$ to a mineral-agar medium efficiently suppresses its contamination under low and medium load,without apparently affecting the growth of mildew or the interaction between mildew and *mlo*-barley on leaf segments placed on the medium.

Keywords: contamination; silver; $AgNO_3$; barley; leaf segments; powdery mildew; *mlo*

In experiments with obligate plant parasites, detached leaf segments on an agar medium are often used under non-sterile conditions: for example, if living spores of powdery mildew or leaf rust are sampled outdoors onto leaf segments in a spore trap, or in inoculation experiments when no laminar air-flow boxes are available. The agar may become accidentally contaminated with airborne bacteria or moulds that sometimes overgrow the sampled parasite on the leaf segments or kill the leaf tissue. Therefore antibiotics are added to the medium in some laboratories. This is costly and only partly solves the problem, since the spectrum of sensitive micro-organisms is limited. An alternative solution would be to utilise the oligodynamic effect of silver ions which kill bacteria within hours at 0.05 to 0.5 ppm (GRIER1983; THURMAN & GERBA1989). Since silver ions are precipitated by Cl-ions, present in the tissue, to non-soluble AgCl and finally reduced by light to nanoclusters of metallic silver, they are probably not taken up by the plant tissue and therefore are not expected to interfere with the studied host–parasite interactions or harm the plant tissue, unlike chemical disinfectants or antibiotics. The aim of the presented experiments was therefore to study the efficacy of different concentrations of ionic silver in suppressing the contamination of a mineral–agar medium and whether or not the growth of powdery mildew on leaf segments or the interactions between the mildew culture and the varieties with or without the *mlo* gene was affected.

MATERIALS AND METHODS

Medium. Petri dishes of 12 cm inner diameter with 60 ml of a medium containing 0.4% agar, 20 ppm benzimidazole (commonly used to retard leaf senescence *in vitro*), 50 ppm of Scherings WUXAL-SUPER® mineral nutrient solution and a variable amount of $AgNO_3$ were used in the experiments. The medium was prepared by suspending agar powder in a small amount of demineralised water, pouring it into hot demineralised water and boiling for 10 minutes. When the solution cooled down to approx 50°C, benzimidazole and mineral nutrients were added from 2000 ppm stock solutions. A stock solution of 1000 ppm $AgNO_3$ was prepared separately, of which 0.0, 0.1, 0.3, 1.0, and 3.0 ml/l were added to the still liquid agar medium. Thus the final concentration of $AgNO_3$ in the dishes was 0.0, 0.1, 0.3, 1 and 3.0 ppm. Three Petri dishes of each Ag-concentration were prepared.

Plant material. In each Petri dish, 25 mm long segments of primary leaves of ten days old seedlings of Diamant, Apex and the Pallas near isogenic lines P8 and P11 were placed on the medium and uniformly inoculated with the partially *mlo*-virulent mildew culture PV-32, described earlier (SCHWARZBACH *et al*. 2002). The varieties, except Apex, do not have the *mlo* gene and are susceptible to the mildew culture PV-32. Cv. Apex has the mlo_{11} allele for resistance against almost all existing mildew pathotypes and is partially resistant to PV-32. The segments were arranged side-by-side in the order P8– APEX–Diamant–Apex–P11–Apex–P8.

Inoculation. The Petri dishes were uniformly inoculated with conidia of PV-32 at a density of approximately 10^3 spores per cm^2 in a settling tower as described earlier (SCHWARZBACH 2001). Three Petri dishes with the same Ag-concentration were inoculated at a time. The uniformity of inoculation was tested by counting the spores within two random microscope view-fields of 2 mm^2 on the agar surface of each Petri dish. The spore counts are summarised in Table 1.

Table 1. Test of inoculation uniformity

$AgNO_3$ (ppm)	Conidia counts*						Mean
0.0	25	19	26	17	14	33	22.3
0.1	17	28	25	21	18	16	20.8
0.3	33	26	17	21	20	17	22.3
1.0	18	25	25	19	14	23	20.7
3.0	18	28	22	20	15	14	19.5

*Conidia counts on the medium in 2 mm^2 view-fields after inoculation in a settling tower

The spore counts were compared with the expected Poisson distribution, in which the variance equals the mean.

The fit was tested by the relation of the observed variance to the mean, using the *F*-test. The calculated *F*-value of 1.33 is far below a significant deviation from the expectation, even at the weak probability level of $\alpha = 0.05$. Therefore, the inoculation can be regarded as fairly uniform, although some small differences between the series cannot be excluded.

Incubation. All series of experiments were incubated in a thermostat at 18°C under continuous light of a 10 W daylight fluorescent tube, fitted 56 cm above the leaf segments.

Conidial germination was examined 24 h after inoculation in two random microscope view-fields of 2 mm^2 in each Petri dish. The error was calculated from the differences between the six view-fields within each Ag-concentration.

Mildew infection was evaluated 6 days after inoculation by counting the number of mildew colonies on each leaf segment, using a 6× magnifying lens. Since there were no significant differences in colony counts between the non-*mlo* barleys, these were pooled together as "*Mlo*-barley", in contrast to cv. Apex as the *mlo*-barley.

Contamination. The open Petri dishes containing the freshly inoculated leaf segments were exposed for 1 h to different contaminating environments. One Petri dish of each Ag-concentration was exposed to laboratory air (lowest load), the second in a humid mouldy basement close to stored potatoes and vegetables (medium load), and the third outdoors on a compost heap of decaying garden and kitchen garbage (heavy load). The dishes were protected against insects by a fine sieve. Since the contaminating load was heavy and very mixed, no determination of the involved micro-organisms was carried out. After 12-days of incubation, the contamination of the medium was examined within each Petri dish under the microscope at a 10 × 10 magnification at 12 spots distributed around the laid out segments. At 6 spots the length of hyphae, relative to the view-field diameter was estimated. At the other 6 spots, the total area of non-hyphal colonies, relative to the view-field, was estimated.

RESULTS

The germination of PV-32 conidia at different concentrations of $AgNO_3$ in the medium is summarised in Table 2. Since there were no significant differences in germination between the different contamination loads, the data from the different loads were pooled together (Fig. 1).

The data show a slight, but significant decrease in spore germination on the agar surface with increasing $AgNO_3$ concentration. This can be expected since, for physical reasons, the damage done by silver ions or nanoparticles to micro-organisms is greater for smaller objects. Since mildew spores are much larger than the contaminant spores, their sensitivity to silver would be

Table 2. The effect of $AgNO_3$ concentration in the medium on the colony number on leaf segments with and without the *mlo* gene, inoculated with the mildew culture PV-32

$AgNO_3$ (ppm)	Low load		Medium load		Heavy load		Mean		*mlo/Mlo* (%)
	mlo	*Mlo*	*mlo*	*Mlo*	*mlo*	*Mlo*	*mlo*	*Mlo*	
0	8.0	51.2	3.3	51.0	6.7	53.7	6.0	52.0	11.5
0.1	4.7	65.5	7.3	69.2	9.7	67.5	7.2	67.4	10.7
0.3	8.0	53.5	6.0	45.5	4.0	67.5	6.0	55.5	10.8
1.0	5.3	60.7	6.3	54.5	3.3	48.7	5.0	54.6	9.2
3.0	5.3	60.0	4.3	56.2	4.3	57.0	4.6	57.5	8.1
Standard deviation of series means (from s^2 within series)							1.12	3.36	**1.93**
Mean	**6.3**	**58.2**	**5.4**	**55.3**	**5.6**	**58.9**	**5.8**	**57.4**	**10.1**

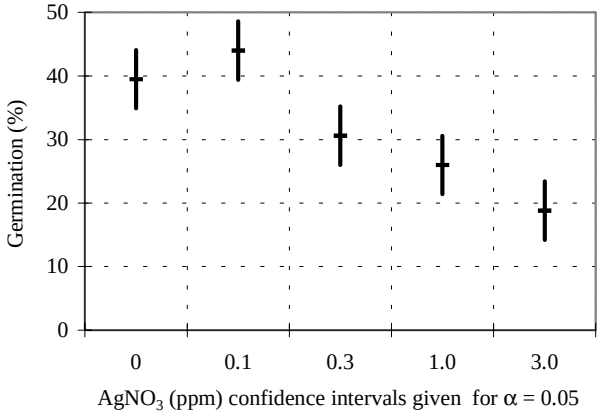

Fig. 1. Conidia germination on the medium with different $AgNO_3$ concentration

expected to be smaller than that of the contaminating micro-organisms.

The effect of silver on infection efficiency and on the interaction between the PV-32 culture and the *mlo* gene is summarised in Table 2.

No significant effects of silver or contamination load on the infection efficiency of PV-32 or mildew colony number on *mlo*-segments, relative to *Mlo*-segments, could be observed. The slight decrease in infection of *mlo*-segments relative to *Mlo*-segments with increasing Ag-concentration was not significant and therefore most likely to be accidental. There is no evidence, therefore, that the addition of $AgNO_3$ to the medium affected the infection efficiency of the mildew culture PV-32 on segments with or without the *mlo* gene.

The effects of silver on contamination of the agar-medium, observed microscopically, are summarised in Tables 3 and 4.

Table 3. The average per cent extension of mould hyphae across microscope view-fields 12 days after exposure to different contaminating environments

$AgNO_3$ (ppm)	Low load (laboratory)	Medium load (humid cellar)	Heavy load (garbage heap)
0.0	51	65	100
0.1	40	83	100
0.3	17	17	87
1.0	0	0	26
3.0	0	0	0

The figures are means of 6 estimates at different spots within the same Petri dish

Table 4. The average per cent coverage of non-hyphal micro-organisms observed in a microscope view-field 12 days after exposure to different contaminating environments

$AgNO_3$ (ppm)	Low load (laboratory)	Medium load (humid cellar)	Heavy load (garbage heap)
0.0	2	65	83
0.1	17	31	20
0.3	0	0	26
1.0	0	0	0
3.0	0	0	0

The figures are means of 6 estimates at different spots within the same Petri dish

The effects of silver are evident from the results in both tables and testing of their statistical significance is not necessary. The visual evaluation of the effects of silver on the contamination of the agar medium, exposed to different contaminating environments, is described in Table 5.

Table 5. Visual evaluation of contamination 12 days after exposure of the agar medium to different contaminating environments

$AgNO_3$ (ppm)	Low load (laboratory)	Medium load (humid cellar)	Heavy load (garbage heap)
0.0	colonies of moulds and other micro-organisms growing abundantly on more than half the surface	crowded colonies of moulds and other micro-organisms growing abundantly on most of the surface	continuous layer of moulds and other micro-organisms on the whole medium surface
0.1	12 distinct colonies with mycelium and dozens of spots of other micro-organisms	14 distinct colonies with mycelium and hundreds of spots of other micro-organisms	dozens of colonies with mycelium and countless spots of other micro-organisms
0.3	4 colonies with mycelium and a few spots of other micro-organisms	6 colonies with mycelum and a few dozen spots of other micro-organisms	a few dozen mycelial colonies and several hundred spots of other micro-organisms
1.0	no contamination	no contamination	90 small colonies not exceeding 3 mm, no mycelium visible
3.0	no contamination, light brownish discoloration of the agar medium	no contamination, light brownish discoloration of the agar medium	3 small colonies below 3 mm, light brownish discoloration of the agar medium

DISCUSSION

The results show clearly that $AgNO_3$ is a powerful means of protecting agar media containing detached leaves against contamination from non-sterile environments. Since NO_3^- is a basic plant nutrient, the effects are due to silver ions or nano-particles of metallic silver. The very light brownish discoloration of the agar medium at 3 ppm is obviously an effect of the photo-reduction of silver ions to nano-clusters of metallic silver and is of no consequence for the experiments. Apart from being very cheap, silver is, according to the US Environmental Protection Agency (EPA), nontoxic and non-carcinogenic. The only undesirable clinical effect of silver is an irreversible skin discoloration (argyrosis) after very high i.v. doses in the order of grams or oral doses in dozens of grams (GAUL & STAUD 1935; FURCHNER *et al*. 1968). If the contamination load is not extremely high, 1 ppm $AgNO_3$ added to the medium protects it safely against contamination. In routine work with this concentration, ocassionally yeasts ocurred on exudates from the cutting edges of the leaf segments, spreading onto the agar medium, or moulds developed from particles of organic impurities or dirt. This is not surprising, since the exudates or particles of impurities are not expected to contain protective amounts of silver.

Acknowledgement: The help of the Research Institute for Plant Production at Prague-Ruzyně, which lent me a microscope, was greatly appreciated. Thanks to JOHN CLARKSON, NIAB Cambridge, for correcting the English.

References

GAUL L.E., STAUD A.H. (1935): Clinical spectroscopy. Seventy cases of generalized argyrosis following organic and colloidal silver medication including a biospectrometric analysis of ten cases. J. Am. Med. Assoc., **104**: 1387–1390.

FURCHNER J.E., RICHMOND C.R., DRAKE G.A. (1968): Comparative metabolism of radionuclides in mammals – IV. Retention of silver – 110m in the mouse, rat, monkey, and dog. Health Phys., **15**: 505–514.

GRIER N. (1983): Silver and its compounds. In: BLOCK S. (ed.): Disinfection, Sterilization and Preservation. Lea & Febiger, Philadelphia: 380–428.

SCHWARZBACH E. (2001): Heat induced susceptibility of *mlo*-barley to powdery mildew (*Blumeria graminis* D.C. f.sp. *hordei* Marchal). Czech J. Genet. Plant Breed., **37**: 82–87.

SCHWARZBACH E., SLATER S.E., CLARKSON J.D.S. (2002): Occurrence of partially *mlo*-virulent isolates of barley powdery mildew in agricultural environments in Europe. Cereal Rusts and Powdery Mildews Bull., **30** [www.crpmb.org/]2001/0208schwarzbach/.

THURMAN R., GERBA C. (1989): The molecular mechanisms of copper and silver ion disinfection of bacteria and viruses. Crit. Rev. Envir. Control., **18**: 295–315.

US Environ. Protection Agency (EPA), document: Silver, CASRN 7440-22-4, updated 1996.

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Received for publication November 16, 2001

Accepted after corrections February 28, 2002

Abstrakt

SCHWARZBACH E. (2002): **Vliv stříbra na mikrobiální kontaminaci agarového média a na interakce mezi padlím a listovými segmenty ječmene s genem *mlo* a bez něho**. Czech J. Genet. Plant Breed., **38**: 82–86.

Segmenty primárních listů linií ječmene lišících se genem *mlo* byly vyloženy v Petriho miskách na agarovém médiu s benzimidazolem, minerálními živinami a s $AgNO_3$ v koncentracích 0, 0,1, 0,3, 1 a 3 ppm. Od každé koncentrace byly připraveny tři Petriho misky. Segmenty byly rovnoměrně infikovány parciálně *mlo*-virulentní kulturou padlí PV-32 v množství 10^3 konidií/cm^2. Poté byla vždy jedna otevřená Petriho miska z každé koncentrace vystavena po 1 hodinu jinému prostředí s odlišným kontaminačním potenciálem: jedna v laboratoři (mírná zátěž), jedna ve vlhkém sklepě blízko skladované zeleniny (střední zátěž) a jedna na hromadě kompostu s rozkládajícími se odpadky (silná zátěž). Klíčení spor padlí na povrchu média mírně klesalo se zvyšující se koncentrací $AgNO_3$. Napadení padlím bylo hodnoceno 7 dní po infekci. Počet kolonií padlí na listových segmentech a interakce kultury padlí s geny *mlo* a *Mlo* nebyly znatelně ovlivněny koncentrací $AgNO_3$. Kontaminace média mikroorganismy ze vzduchu byla hodnocena mikroskopicky i vizuálně 12 dní po expozici a narůstala se zátěží prostředí a se snižující se koncentrací $AgNO_3$. Koncentrace 0,1 ppm $AgNO_3$ zřetelně omezila růst kontaminujících kolonií, ale nezabránila kontaminaci v žádném prostředí. Při koncentraci 1 ppm $AgNO_3$ nebyla pozorována kontaminace média vystaveného mírné nebo střední zátěži, avšak desítky malých kontaminujících kolonií se vyvinuly na médiu vystaveném silné zátěži. Při koncentraci 3 ppm $AgNO_3$ vznikly pouze tři malé kontaminující kolonie na médiu vystaveném silné zátěži, zatímco média vystavená střední a mírné zátěži zůstala čistá. Lze učinit závěr, že přidání 1 ppm $AgNO_3$ k médiu s minerálním agarem účinně zabraňuje jeho kontaminaci za nesterilních podmínek při mírné a střední zátěži, avšak neovlivňuje na listových segmentech, vyložených na médiu, růst padlí ani interakci padlí s geny *Mlo* a *mlo*.

Klíčová slova: kontaminace; stříbro; $AgNO_3$; ječmen; listové segmenty; padlí travní; *mlo*

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