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Tolerance to agricultural pesticides of strains belonging to four genera of *Rhizobiaceae*

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In order to determine their tolerance to pesticides, 122 strains of rhizobia isolated from different geographical regions, and belonging to the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Bradyrhizobium* were tested against eight herbicides, four fungicides and five insecticides. Sensitivity to the pesticides was measured by using the filter paper disk method at four concentrations, 0.45, 4.5, 45 and 450 μg per disk. When the pesticides were used at 0.45 μg per disk, a concentration similar to that found when pesticides are applied under field conditions, no inhibition was observed. Strains growth was affected at concentrations of 45 and 450 μg pesticide per disk. These higher concentrations can be encountered when seeds are treated with pesticides. Pesticides tolerance level was correlated to pesticide function, *i.e* rhizobial strains were more tolerant to insecticides, followed by herbicides and then fungicides. Two fungicides, captan and mancozeb, inhibited the highest number of strains. Only one insecticide, carbaryl, affected the growth of some rhizobial strains. Strains isolated from the arctic (*Mesorhizobium* spp. and *R. leguminosarum* bv. viciae), a putative pesticides-free environment, were either less or equally affected by pesticides compared to strains isolated from agricultural regions.

Keywords: *Rhizobium*; *Bradyrhizobium*; arctic rhizobia; fungicides; herbicides; insecticides.

Introduction

When applied to soil, plant foliage or directly on seeds, pesticides can enter the soil environment by direct application or via plant root exudates. This input of pesticides can affect many soil organisms in different manners. Even though some soil bacteria are able to tolerate or degrade some pesticides by using them as their sole carbon or nitrogen source,[1−3] bacteriostatic and lethal effects can also occur.[4]

As a group, rhizobia are mainly known for their ability to form N_2-fixing nodules in symbiosis with legumes, supplying most of the plant need in nitrogen. Any adverse effect on rhizobia affects the legume-rhizobium symbiosis by reducing the rate of biological nitrogen fixation, which can decrease plant productivity. Previous studies reported mixed effects of the different classes of pesticides (insecticides, herbicides and fungicides) on rhizobia. Some pesticides were shown to inhibit rhizobial growth[5−8] or legume nodulation,[9−11] while others had no adverse effects.[7,10,11] The effect of pesticides on rhizobia and their symbiosis with legume, will vary according to the rhizobial species,[12−14] the rhizobial strains within a given species,[5,7,15−18] the type of pesticide involved,[14,16,17,19] and the pesticide concentration.[9,15,20]

Published studies regarding pesticides' effects on free living or symbiotic rhizobia are usually scattered and done with few pesticides on a limited number of strains[7−9,11,20,21] or with a limited number of pesticides on different strains of the same genus.[5,7,15,19,22] To our knowledge, no study investigated a combination of many pesticides with many rhizobial strains belonging to different genera and species. The aim of the present study was to evaluate the intrinsic resistance of 122 rhizobial strains belonging to four genera (*Rhizobium, Sinorhizobium Mesorhizobium, Bradyrhizobium*), to 17 different pesticides (herbicides, insecticides and fungicides). Although bacteria are more sensitive to pesticides in pure culture studies as compared to *in situ* enumeration techniques,[9] the results reported in the present study will supply information that will be useful to predict the response of legumes to rhizobial inoculation in the presence of pesticides. Strains isolated from both agricultural and arctic ecosystems

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were included in order to differentiate intrinsic genetic resistance from adaptation to environments exposed to pesticides.

Materials and methods

Bacterial strains

A total of 122 strains of rhizobia from the culture collection of Agriculture and Agri-Food Canada (Québec, Canada) were used (Table 1). They were selected in order to get a representative spectrum of different genera, species and geographic origins. Some strains originated from remote arctic regions presumably not exposed to pesticides while others were isolated from agricultural areas where pesticides are used. The strains belonged to the genera *Rhizobium* (44 strains), *Mesorhizobum* (21 strains), *Sinorhizobium* (24 strains) and *Bradyrhizobium* (33 strains). Before the start of the experiment, the purity of each strain was verified on yeast extract mannitol agar (YMA) with Congo Red (0.1 %).[23]

Pesticides tested

The active ingredients of eight herbicides, four fungicides and five insecticides were used (Table 2). Stock solutions of 5000 μg mL^{-1} of the active ingredient from each pesticide were prepared in different solvents, depending on their solubilities (Table 2). The stock solution of each pesticide was further diluted to 500, 50 and 5 μg mL^{-1} using the same solvent. Because the molecules had different molecular weights, this resulted in different molar concentrations for each pesticide, as indicated in table 2 for the 5000 μg mL^{-1} concentration.

Disc diffusion method

Sensitivity levels of the different strains to selected pesticides were determined using the filter paper disk technique, designed to test bacterial antibiotics resistance[24] and later used to test the effect of pesticides on bacterial growth.[7,25] Ninety μL of each pesticide solution were applied on a sterile 12.5 mm diameter filter paper disk (Schleicher & Schuell, USA) to obtain a final amount of 450, 45, 4.5, and 0.45 μg of pesticide per disk. Because of the different molecular weights of the pesticides' active ingredients (Table 2), the number of moles per disk varied from one pesticide to the other, for the same amount added. For example, at a level of 450 μg per disk, the number varied from 1.11 mmol per disk for endosulfan (highest molecular weight) to 2.66 mmol per disk for glyphosate (lowest molecular weight). This variable was considered in the interpretation of results. The wet disks were dried in a sterile biological hood for 24 hours at room temperature to remove solvents and stored at 4°C under nitrogen for a maximum of four weeks. Control disks with no pesticide were prepared with the different solvents to take into account any residual solvent effect. Considering the surface area of the filter paper used and the different concentrations of pesticide solutions, the calculated rate applied ranged from 0.367 kg ha^{-1} (0.45 μg per disk) to 367 kg ha^{-1} (450 μg per disk).

Bacterial strains were grown in yeast extract mannitol (YM) broth[26] until they reached mid-exponential growth phase ($OD_{600} = 0.8$). A sterile cotton swab was dipped into the cell culture and used to uniformly inoculate the surface of YMA plates. Pesticide disks were positioned on the agar surface, in the center of each quadrant. Each plate thus contained four disks, which corresponds to the four concentrations prepared for each pesticide. The plates were kept overnight at 4°C to allow diffusion of the pesticide and incubated at 28°C for at least five days, depending on the growth rate of strains. For each disk, determination of the inhibition zone was made from two perpendicular measurements. Inhibition was considered positive when the diameter of the inhibition zone was more than 2 mm larger than the diameter of the paper disk. Strains *Mesorhizobium loti* NZP 2234 and *Bradyrhizobium lupini* T12 were used as controls in each batch to check for variability resulting from the preparation of media or pesticide disks.

Results and discussion

Pesticide concentration

When applied at 0.45 and 4.5 μg per disk, no growth inhibition was observed with the 122 strains tested. These two concentrations are equivalent to application rates of 0.367 and 3.67 kg ha^{-1} respectively, and they are representative of the amount of pesticides remaining in the soil environment following applications. These results suggest that the tested rhizobia, including arctic strains, tolerate the pesticides used at concentrations similar to those found under field conditions. At 45 μg per disk, only the two fungicides captan and mancozeb inhibited the growth of a great number of strains in each genera (21 to 100 % of the tested strains) while the two herbicides atrazine and paraquat were bactericidal to only a few strains of *Mesorhizobium* (5 %) and *Bradyrhizobium* (Table 3). The inhibitory effect was more pronounced at 450 μg per disk, as shown by the higher number of strains sensitive to seven herbicides, four fungicides and one insecticide (Table 3). Strains susceptible to a pesticide at 45 μg per disk were also inhibited by 450 μg per disk of the same pesticide. The size of the inhibition zone increased when higher pesticide concentrations were used (data not shown). The increase in the size of the inhibition zone can be an indication of higher toxicity,[7] or can result from a greater diffusion of the pesticide in the

Table 1. Source and origin of the rhizobial strains used in this study.

Genus	Species	Number of strains	Strains	Geographic origin
Temperate agricultural strains				
Rhizobium	*leguminosarum* bv. viciae	18	USDA 2445	South Carolina, USA
			USDA 2489 (Nitragin 175F16)	Unknown
			USDA 2370 ^{T}	Illinois, USA
			CBh5, CBp7, CBa1, CBe6	Normandin (Québec), Canada
			CB3, CB13	St-David (Québec), Canada
			Lp0101, Lp0107, Lp0204, Lp0501, Lp0606, Lp0610, Lp1009	Abitibi (Québec), Canada
			175P1 (Nitragin)	Unknown
			FS-163-Ka	Québec, Canada
	leguminosarum bv. phaseoli	3	FCSV F2	Ste-Foy (Québec), Canada
			FEV Ju L1, FEV Ju L2	Normandin (Québec), Canada
	leguminosarum bv. trifolii	14	TL-4, TL-5, TL-6, TL-10, TL-11, TL-12, TL-13	Québec, Canada
			FH-345-K, FH-20-SI, F-134-S, F-131-Sp, FH-13	Québec, Canada
			RL2, RL3	Québec, Canada
	etli	1	CFN42 ^{T}	Mexico
	tropici	1	CIAT 899 ^{T}	Columbia
Mesorhizobium	*loti*	6	L2, L3, L4, L5	Québec, Canada
			USDA 3471^{T}, NZP 2234^{T}	New Zealand
	ciceri	1	UPM-CA7 ^{T}	Spain
	sp.	6	9B2, 9B5 (Nitragin)	Alberta, Canada
			AA1, AA2	Alberta, Canada
			USDA 3549, USDA 4003	Alaska, USA
Sinorhizobium	*meliloti*	23	3Doa20a (USDA1020a),	North Dakota, USA
			3Doa8 (USDA1008)	Maryland, USA
			A1, A2, A3, A4, A5, D1, D2, D3, S1, S10, S11, S14, S28, S33, S46, V2, V6, V7	Québec, Canada
			USDA 1002 ^{T}, ATCC 9930 ^{T}	North Virginia, USA
			AlfalfaD	Québec, Canada
	fredii	1	USDA 191 ^{T}	China
Bradyrhizobium	*japonicum*	20	1Ma2, 3Ma2, 5Ma1, 7Ma1, 4Mb1, 1Sb2, 3Sa2, 5Sb1, 6Sa1, 7Sa2	Québec, Canada
			61A-101C, 61A-124a (Nitragin),	Unknown
			61A-148 (Nitragin)	India
			JPD1a, SGR1, SPE2	Québec, Canada
			532C (Nitragin 61A152)	Unknown
			TOPP1a, TOPP5a	Ontario, Canada
			ATCC 10324 ^{T}	Japan
	elkanii	1	USDA 76 ^{T}	California, USA
	lupini	12	LupinSulfa, LupinWelko	Normandin (Québec), Canada
			LL13, LL15, LL90, T12, LB84	
			Nit96E3 (Nitragin)	Unknown
			USDA 3046, USDA 3051, ATCC 10317, ATCC 10319 ^{T}	Georgia, USA
Arctic strains				
Rhizobium	*leguminosarum* bv. viciae	7	Lj3, Lj8, Lj15, Lj20, Lj25, Lj27, Lj30	Kuujjarapik (Québec), Canada
Mesorhizobium	sp.	8	N1, N13, N25, N31, N33, N38, N39, N40	Scarpa Lake, Nunavut, Canada

[1]T: type strains; USDA : United States Department of Agriculture; NZP : Division of Scientific and Industrial Research, New Zealand; ATCC : American Type Culture Collection. Nitragin: EMD Crop BioScience, Wisconsin, USA. Other abbreviations are strains designations of the culture collection of AAFC (Agriculture and Agri-Food Canada) Research Centre, Québec.

Table 2. Dosage and the solvents used for preparation of the active ingredient of the different pesticides tested[1].

Type	*Active compound*	*Solvent used*	*Molecular weight ($g\ mol^{-1}$)*	*Molar concentration at 5000 $\mu g\ mL^{-1}$ ($mmol\ mL^{-1}$)*
Herbicides				
	Atrazine	Methanol	215.69	23.18
	Dicamba	Ethanol	221.04	22.62
	Glyphosate	Water (distilled)	169.08	29.57
	Imazethapyr	Methanol	289.30	17.28
	Linuron	Methanol	249.11	20.07
	Metolachlor	Methanol	283.80	17.62
	MCPA	Ethanol	200.62	24.92
	Paraquat	Water (distilled)	257.20	19.44
Fungicides				
	Benomyl	Ethanol	290.62	17.20
	Captan	Acetone	300.61	16.63
	Mancozeb	Dichloromethane	266.31	18.78
	Thiram	Acetone	240.44	20.80
Insecticides				
	Azinphos methyl	Dichloromethane	317.32	15.76
	Carbaryl	Methanol	201.23	24.85
	Carbofuran	Acetone	221.25	22.60
	Chlorpyrifos	Methanol	350.62	14.26
	Endosulfan	Ethanol	406.96	12.29

[1]The different properties were obtained from EXTOXNET (http://extoxnet.orst.edu).
Choice of a solvent was based on the optimal solubility in the concentration range selected for the experiments.

agar far from the paper disk due to the higher concentration gradient.

Herbicides

The 122 rhizobial strains tested were not inhibited by the herbicides dicamba, glyphosate, imazethapyr, linuron, metolachlor and MCPA when used at 45 μg per disk.

At this concentration, atrazine inhibited only one strain of *Mesorhizobium* while paraquat inhibited three strains of *Bradyrhizobium* (Table 3). The 45 μg per disk concentration is equivalent to an application rate of 36.7 kg ha^{-1}, which is about 10 times the regular amount of atrazine and paraquat applied under field conditions according to the application rates found on commercial product labels of these two herbicides.

At 450 μg per disk, seven of the eight herbicides tested induced growth inhibition in at least one genus of rhizobia (Table 3). The herbicide imazethapyr had no effect on all strains tested. Our results corroborate those of Gonzalez et al.[10] who did not find any direct effect of this herbicide on *Rhizobium* species. They attributed the decrease in pea nodulation following imazethapyr treatment to an effect on plant growth rather than on rhizobia. According to Royuela et al.,[27] tolerance of *Rhizobium* to imazethapyr is related to their high acetolactate synthase (ALS) activity, which counteract the herbicide mode of action (ALS inhibition).

Metolachlore and paraquat did not exhibit any bactericidal effect on all *Sinorhizobium* strains tested, and similarly metolachlore and atrazine did not affect any of the *Rhizobium* strains (Table 3). Bouquard et al.[28] showed that strain PATR of *Rhizobium* sp. isolated from an agricultural soil previously treated with atrazine was able to degrade this herbicide by dehalogenation. According to these authors, members of the genus *Rhizobium* are likely to play an important role in the biological degradation of atrazine. The ability of *Rhizobium* strains to degrade herbicides can explain their tolerance of very high concentrations. In our study, *Mesorhizobium* tolerated dicamba and MCPA at 450 μg per disk, while *Bradyrhizobium* strains were resistant to atrazine, glyphosate and MCPA. Linuron was the most toxic herbicide for *Sinorhizobium* and *Bradyrhizobium*, as shown by the higher number of strains inhibited in comparison to other herbicides. For *Mesorhizobium*, atrazine inhibited the highest number of strains (58 %), while linuron and MCPA affected the same number (14 %) of *Rhizobium* strains (Table 3). Moorman[16] found that growth of *B. japonicum* strains 110SK and 138ES in yeast extract manitol (YM) broth was not affected by exposure to 2 and 20 mg L^{-1} glyphosate, 2 mg L^{-1} linuron or 1 and 10 mg L^{-1} paraquat. Growth of strain 110SK was significantly reduced in the presence of 20 mg L^{-1} linuron. Mallik and Tesfai[7] also found that glyphosate had no effect on the growth of a strain of *B. japonicum* (*R. japonicum*) at concentrations up to 25 mg mL^{-1} in YM. According to Moorman,[16] YM broth supplied enough amino acids to counteract the effect of pesticides such as glyphosate and paraquat, acting as amino acid inhibitors in bacteria. In

Table 3. Number of strains within each rhizobium genus that are sensitive to different pesticides at concentrations of 45 and 450 μg per disk[1].

	Sinorhizobium *24 strains*		Rhizobium *44 strains*		Mesorhizobium *21 strains*		Bradyrhizobium *33 strains*	
Type	***45 µg***	***450 µg***	***45 µg***	***450 µg***	***45 µg***	***450 µg***	***45 µg***	***450 µg***
Herbicides								
Atrazine	0	1 (4 %)	0	0	1 (5 %)	12 (58 %)	0	0
Dicamba	0	1 (4 %)	0	2 (5 %)	0	0	0	1 (3 %)
Glyphosate	0	2 (9 %)	0	1 (2 %)	0	2 (10 %)	0	0
Linuron	0	6 (26 %)	0	6 (14 %)	0	2 (10 %)	0	22 (67 %)
MCPA	0	2 (9 %)	0	6 (14 %)	0	0	0	0
Metolachlore	0	0	0	0	0	1 (5 %)	0	1 (3 %)
Paraquat	0	0	0	4 (9 %)	1 (5 %)	1 (5 %)	3 (9 %)	7 (22 %)
Fungicides								
Benomyl	0	0	0	1 (2 %)	0	1 (5 %)	0	1 (3 %)
Captan	22 (92 %)	24 (100%)	43 (98 %)	43 (98 %)	21 (100%)	21 (100%)	30 (91 %)	32 (97 %)
Mancozeb	5 (21 %)	24 (100%)	10 (23 %)	43 (98 %)	14 (67 %)	21 (100%)	30 (91 %)	33 (100%)
Thiram	0	9 (38 %)	0	7 (16 %)	0	3 (14 %)	0	10 (30 %)
Insecticides								
Carbaryl	0	0	0	6 (14 %)	0	2 (10 %)	0	10 (30 %)

[1]Only the pesticides having an inhibitory effect are presented.
MCPA:

this study, the YM agar used did not prevent growth inhibition of some strains by glyphosate (450 μg per disk) and paraquat (45 and 450 (μg per disk).

Fungicides

Captan at both concentrations tested was the fungicide that affected the highest number of strains belonging to the four genera, with 100 % of *Mesorhizobium* strains inhibited and 91 to 100 % of *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium* strains (Table 3). Captan was previously shown to be toxic to different strains of *Rhizobium*,[6,29] and *Bradyrhizobium*.[7,19,30,31] The fungicide mancozeb similarly affected almost all strains at 450 μg and especially strains of *Mesorhizobium* (67 %) and *Bradyrhizobium* (91 %). When applied at a concentration of 45 μg per disk, strains of *Rhizobium* (21 %) and *Sinorhizobium* (23 %) were less affected by mancozeb than *Mesorhizobium* (67 %) and *Bradyrhizobium* (91 %). Thiram was toxic only at 450 μg per disk for few strains (14 to 38%) within each rhizobial genus. Curley and Burton[30] showed that thiram applied as a soybean seed protectant at 0.6 g kg^{-1}, did not show any adverse effect on the survival of *Bradyrhizobium japonicum* from a peat-base inoculums. However, Mallik and Tesfai.[7] using a paper disk test similar to the one used in this work found that strain LU1 of *B. japonicum* was highly sensitive to thiram.

Benomyl was the less toxic fungicide. In fact, only when used at a concentration of 450 μg per disk it inhibited the growth of the two strains 9B5 of *Mesorhizobium* and TOPP1a of *Bradyrhizobium* (Tables 4 and 5). In an experiment studying the effects of four fungicides on soil microbial populations, Ojo et al.[32] found more bacteria in samples treated with benomyl as compared with the other fungicides. Benomyl is readily degraded to carbendazim, which is another fungicide[33] Carbendazim was shown to be very toxic to rhizobia.[19] Since little growth inhibition was observed in the present study, it is possible that the transformation of benomyl to carbendazim did not occur under our experimental conditions.

The mode of action of captan is classified as M4 according to the Fungicide Resistance Action Committee (FRAC) classification, while mancozeb is classified as an M3 fungicide. Fungicides belonging to M3 and M4 mode of action classes are multi–site contact fungicides and affect the target pest at non-specific sites. As far as pesticide resistance is concerned, multi–site pesticides are good because it is more difficult for a given organism to develop resistance strategies against the pesticide. On the other hand, these pesticides may be less specific regarding the type of organisms they can affect, as shown in the present study. Thiram is also an M3 fungicide belonging to the same chemical family as mancozeb (dithiocarbamates). However, it had a less pronounced effect on rhizobial growth inhibition than mancozeb. Even if the stock solution of mancozeb and thiram were prepared in different solvents (Table 2), control plates treated with solvent showed no growth inhibition on any of the strains. A chemical interaction between solvent and pesticide affecting bacterial growth cannot be ruled out. However, it is most likely that, even though the

Table 4. Sensitivity patterns of strains of rhizobia affected by more than one class of pesticides (herbicides, fungicides and insecticides).

	HERBICIDES							*FUNGICIDES*				*INS.*
	Atrazine	*Dicamba*	*Glyphosate*	*Linuron*	*MCPA*	*Metolachlore*	*Paraquat*	*Benomyl*	*Captan*	*Mancozeb*	*Thiram*	*Carbaryl*
Temperate strains												
***Rhizobium* (8/37)**[1]												
USDA 2370				*	*				**	*		*
175P1					*				**	*		*
USDA 2489				*	*				**	**		
TL-5				*			*		**	**	*	*
TL-10				*	*				**	*		*
TL-12		*	*	*			*		**	*	*	*
FH-20-SI				*	*				**	*		*
TL-13					*		*		**	**	*	
***Sinorhizobium* (9/24)**[1]												
A1, USDA191				*					**	**		
A2	*			*					**	*	*	
A3				*	*				**	*	*	
A5		*	*						**	*		
D1				*					**	*	*	
S14				*	*				*	*		
USDA 1002			*						**	*		
FH-13							*		**	*	*	
***Mesorhizobium*(7/13)**[1]												
9B2	*		*	*		*	*		**	**		*
9B5	*		*	*				*	**	**	*	*
AA1, L5, UPM-Ca-7	*								**	**		
USDA 4003	**								**	**	*	
L4	*								**	*		
***Bradyrhizobium* (17/33)**[1]												
5Ma1				*			*		**	**		*
1Sb2				*					*	**	*	*
7Sa2, Nit96E3				*			*		**	**	*	*
TOPP1a				*		*	**	*	**	**	*	*
LL90				*					**	**		*
T12				*					**	*	*	*
1Ma2, 4Mb1,				*					**	*		
3Ma2		*		*			**		**	**	**	
5Sb1,61A-148, SGR1, LL13, USDA3051				*					**	**		
LL15, LB84				*					**	*		
USDA 3046				*			*		**	**		
Arctic strains												
***Rhizobium* (1/7)**[1]												
Lj 25		*							**	**		
***Mesorhizobium* (5/8)**[1]												
N1, N31, N33	*								**	**		
N39, N40	*								**	*		

*growth inhibited with 450 μg of pesticide per disk.
**growth inhibited by 45 and 450 μg of pesticide per disk.
[1]number sensitive strains/total number of strains tested.

Table 5. Number of rhizobial strains sensitive to fungicides and tolerant to the other pesticides tested.

	Benomyl		*Captan*		*Mancozeb*		*Thiram*	
	45 μg	*450 μg*	*45 μg*	*450 μg*	*45 μg*	*450 μg*	*45 μg*	*450 μg*
Temperate strains								
Rhizobium **(27/37)**[1]	0	1[2]	27	27	5	27	0	3
Sinorhizobium **(16/24)**	0	0	16	16	3	16	0	6
Mesorhizobium **(6/13)**	0	0	6	6	3	6	0	0
Bradyrhizobium **(11/33)**	0	0	8	10	2	11	0	0
Arctic strains								
Rhizobium **(6/7)**	0	0	6	6	2	6	0	0
Mesorhizobium **(3/8)**	0	0	3	3	2	3	0	0

[1] Number of strains sensitive only to fungicides over the total number of strains tested.
[2] Number of sensitive strains.

two fungicides belong to the same chemical family and the same mode of action class, their effect on bacterial growth is chemical-specific, resulting in a different mode of action of each fungicide on the different bacterial strains. Our findings of rhizobia sensitivity to fungicides corroborate other published results.[29,34] Fungicides' sensitivity is of particular importance considering their negative effects on the survival of rhizobia on seeds treated with fungicide before sowing.[4,6] Captan and mancozeb with their strong inhibitory effects on rhizobia, should be carefully used with legumes to avoid contact that can cause detrimental effects on nodulation and biological nitrogen fixation.

Insecticides

All strains tolerated the five insecticides tested at 45 μg per disk. At 450 μg per disk, only carbaryl inhibited the growth of rhizobial strains, and the percentage of sensitive strains was rather low: 10 % in *Mesorhizobium,* 14 % in *Rhizobium* and 30 % in *Bradyrhizobium* (Table 3). Kapusta and Rouwenhorst[15] observed that strains of *B. japonicum* grown in pure culture, had different sensitivities to carbaryl. The growth of strains USDA 117 and USDA 123 was completely inhibited at 40 mg L^{-1}, while strain USDA 31 was not affected by carbaryl. Strain AC100 of *Rhizobium* sp. was shown to be able to degrade carbaryl,[35] which can explain part of the genus-specific response towards this pesticide.

The other insecticides tested in the present study (azinphos-methyl, carbofuran, chlorpyrifos and endosulfan) had no inhibitory effect on any of the 122 strains. Mixed results are reported in the literature on the effect of these insecticides on rhizobia. Carbofuran did not cause any harmful effect on symbiotic nitrogen-fixing bacteria, even when they were exposed to high concentrations.[36] Shafiani and Malik[13] found that some *Rhizobium* isolates were inhibited by 100 mg mL^{-1} of carbofuran and endosulfan in liquid growth media when they were applied as commercial grade products. Khan et al.[11] found that chlorpyrifos decreased the number of chickpea *Rhizobium* in the rhizosphere by 90 % when applied as the commercial product Pyrifos 40 % EC (emulsifiable concentrate), while no effects were observed when it was applied as Lorsban 40 % EC. These results may be explained by the different doses of each product applied (1125 mL $acre^{-1}$ for Lorsban as compared to 875 mL $acre^{-1}$ for Pyrifos) or by the presence of products other than the pesticide in the commercial formulations.

Strain/genus specific responses to pesticides

Detailed pesticides sensitivity patterns of the different agricultural and arctic strains of rhizobia are presented in table 4. Regardless of the environment from which they were isolated, none of the strains tested was affected only by herbicides. In fact, strains influenced by at least one herbicide were also affected by at least one fungicide. A total of 14 strains belonging to *Rhizobium, Mesorhizobium* and *Bradyrhizobium* were inhibited by the three groups of pesticides: the insecticide carbaryl and one or more herbicides and fungicides. Some strains were affected by as many as eight different pesticides, like strain TL-12 of *R. leguminosarum* bv. trifoli, strains 9B2 and 9B5 of *Mesorhizobium* sp. and strain TOPP1a of *B. japonicum*. Among *Bradyrhizobium* strains, strain 3Ma2 was affected by the greatest number of pesticides: three herbicides (linuron, paraquat and dicamba) and three fungicides (captan, thiram and mancozeb). The genus *Mesorhizobium* was the only one showing a high proportion of strains susceptible to atrazine when applied at 450 μg per disk regardless of its geographic origin (temperate or cold environments).

It is interesting to note that more than 56 % of strains (69/122) belonging to all rhizobium genera from arctic or temperate regions were affected only by fungicides (Table 5). The majority of strains were affected by the two fungicides captan and mancozeb. The fungicide benomyl affected only one strain of *Mesorhizobium* 9B5 and one strain of *Bradyrhizobium* TOPP1a (Table 4). Among all strains tested, *Rhizobium leguminosarum* bv. viciae Lp 0606 was the only one resistant to all pesticides tested. This strain was isolated from a forage plant, *Lathyrus pratensis*,

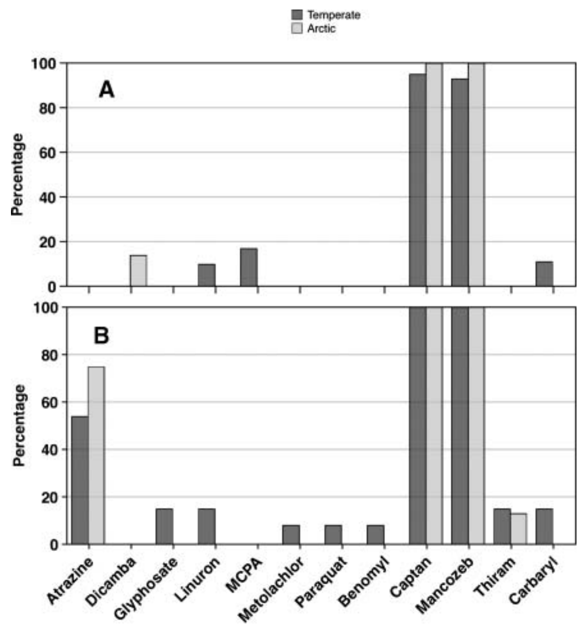

Fig. 1. Susceptibility of *Rhizobium leguminosarum* bv. viciae (A) and *Mesorhizobium* spp. (B) strains from temperate and arctic origin to different pesticides when applied at 450 μg per disk. A total of 25 *R. leguminosarum* bv. viciae (18 temperate, 7 arctic) and 15 *Mesorhizobium* sp. (7 temperate, 8 arctic) strains were tested.

introduced in the Abitibi region (Québec, Canada) around the middle of the 20th century and presently cultivated under low input practices. *Bradyrhizobium japonicum* TOPP5a isolated from a cultivated soybean soil in Ontario was inhibited only by the fungicide mancozeb.

Strains geographic origin

In this work, strains of the genera *Rhizobium* and *Mesorhizobium* isolated from the arctic environment were probably not exposed to pesticides, due to their relative distance from agricultural regions and low possibility of contamination from aerosols dispersion of chemicals by the dominant wind patterns. Comparing the tolerance to pesticides between the arctic and agricultural strains allows some insight into the genetic diversity of tolerance to pesticides. Arctic strains belonging to *R. leguminosarum* bv. viciae were isolated along the coast of the Hudson Bay area in Northern Québec, Canada.[37] The majority of arctic strains (6/7) and temperate agricultural (14/18) strains were susceptible to only the two fungicides captan and mancozeb. However the temperate strains 175P1 and USDA 2370 were sensitive respectively to four and five pesticides (herbicides, fungicides and insecticides). The only strain of *R. leguminosarum* bv. viciae (USDA 2370) affected by linuron, MCPA and carbaryl was isolated from agricultural regions, while Lj 25 isolated from the arctic was the only strain affected by dicamba (Fig. 1A).

The eight arctic strains belonging to the genus *Mesorhizobium* sp. were isolated from the Melville Peninsula in the Canadian high arctic.[38] As observed for strains of *R. leguminosarum* bv. viciae, the eight *Mesorhizobium* arctic strains were affected by fewer pesticides than their temperate counterpart, comprising 13 strains (Fig. 1B). The “arctic” *Mesorhizobium* sp. strains were slightly more sensitive to atrazine (as evaluated by the size of the inhibition zone and the proportion of strains inhibited) than their “agricultural” counterparts (data not shown). Although the number of arctic strains tested was lower than that of temperate strains for both rhizobial species *R. leguminosarum* bv. viciae (7 arctic, 18 temperate) and *Mesorhizobium* sp. (8 arctic, 13 temperate), our results suggest that strains of the same species can respond differently to the presence of pesticides, depending on their geographical origin.

Conclusion

This work shows that fungicides had the highest deleterious effects on the rhizobia, followed by herbicides and then insecticides. The two fungicides that affected the higher number of strains were captan and mancozeb. However, the pesticide concentrations inhibiting growth were much higher than the concentrations expected to come into contact with the bacteria following normal field application, except in the case of seed treatment. We identified one rhizobial strain that was not affected by any of the pesticides tested at any concentration (*Rhizobium leguminosarum* bv. viciae strain Lp 0606) and one strain that was affected only by mancozeb at 450 μg per disk (*B. japonicum* strain TOPP5a).

Our observations suggest that the tolerance to pesticides is probably mainly encoded in the genome of the studied rhizobial species and the evolutionary process could be the principal long time agent that had induced phenotypic differences between the studied genera. For example, only strains of the genus *Sinorhizobium* were not affected by insecticides, while many strains of all other genera were affected by herbicides, fungicides, and insecticides. Furthermore, there were variations from one strain to another within the same genus and these variations seem to follow the usual phenotypic variability observed among strains for other phenotypic traits, like antibiotic resistance.[39] Strains of *R. leguminosarum* bv. viciae and *Mesorhizobium* from arctic regions showed a tendency to be less affected by pesticides than those from temperate regions, however further studies involving a larger number of arctic strains is necessary to better elucidate the effects of geographic regions on the resistance of rhizobia to pesticides.

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