Downy Mildew Tests on Young Sunflower Plants Grown *in Vitro*

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Abstract

The aim of the study was to determine whether it would be possible to evaluate, *in vitro*, the downy mildew resistance of sunflower seedlings obtained from culture of immature embryos. Successful axenic culture of *Plasmopara halstedii* was obtained by growing surface sterilized 10-day-old seedlings that had been infected with downy mildew, in sterile conditions. Two methods were used to multiply the axenic fungus, infection of healthy sunflower callus by downy mildew and callus production from infected seedlings, but neither gave any sporulation at 18°C or 24°C. Only the callus obtained from infected seedlings contained some mycelium and haustoria. *In vitro* tests on mature seed indicated that plants showed the same resistance / susceptibility reaction as under normal conditions. Infection of susceptible immature embryos gave only 50% of plants showing symptoms. Microscopical observations showed that plants without symptoms either contained no *P.halstedii* mycelium or only small quantities. The reliability of *in vitro* downy mildew tests is thus closely linked to successful infection. However, improvements of some parameters should make it possible to carry out downy mildew tests during *in vitro* culture of sunflower embryos.

Introduction

The appearance of new, more virulent races of downy mildew (*Plasmopara halstedii*) in France (Tourvieille de Labrouhe *et al.*, 1988) has made it necessary for breeders to introduce new resistance genes into modern sunflower hybrids. Backcrossing procedures require at least 7 generations, so, in order to accelerate fixation of resistant inbreds, *in vitro* culture (CIV) of immature embryos (Alissa *et al.*, 1986) has been used. This eliminates the period taken by seed maturation and dormancy. Embryos removed from capitula 8 to 20 days after fertilization complete their cycle in about 2 months.

Optimum use of CIV in resistance backcrossing programmes is only possible if susceptible plants can be eliminated at each cycle. The whole seedling immersion method (WSI) of Cohen and Sackston (1973) cannot be used in CIV since germinated seeds are infected in non-axenic conditions. The development of a downy mildew resistance test *in vitro* requires the production of axenic inoculum (zoosporangia). The first work on this was reported by Viranyi and Sziraki (1986) who obtained axenic sporangia after surface sterilization of cotyledons of diseased seedlings. Techniques involving co-culture of plant and parasite from roots obtained after inoculation with *Agrobacterium rhizogenes* were proposed by Zahka and Viranyi (1991).

The aim of this study was to develop the large scale production of downy mildew zoosporangia in axenic conditions and to determine the best conditions for an *in vitro* test on young plants obtained from immature embryos.

Material and methods

Fungal isolates : Two isolates representing two downy mildew races in France were used :
CL 153 : race A, collected in the Indre department in 1988.
CL 152 : race B collected in the Gers department in 1990

Sunflower genotypes : the following, all maintained or produced by INRA or Rustica-Prograin Génétique (RPG) :

Populations :	Peredovik, susceptible to all downy mildew races
	HAR5 (USDA), resistant to race A, susceptible to race B
Hybrids :	TP4 (exp.RPG), susceptible to races A and B
	FHS (exp.RPG), susceptible to races A and B
	Phoebus (var.RPG), susceptible to races A and B
	GH*RHA266(exp.INRA), susceptible to races A and B
	H1 (exp.RPG), resistant to races A and B
Inbreds :	QPH1 (INRA), resistant ro race A, susceptible to race B
	PMI3 (INRA), resistant to race B, susceptible to race A

Experimental conditions : A growth chamber with 14h light per day, at an intensity of 80µE/m²/s. The temperaure was 18°C to 24°C, according to trial.

Immature embryo culture : The method was that described in detail by Pelletier *et al.* (1995). Achenes were removed from capitula 10 to 20 days after fertilization, when the embryos measured 5 to 8 mm. They were disinfected in 12% sodium hypochlorite solution for 5 min., rinsed 3 times in distilled water, then hulled in sterile conditions. The growth media were those of Alissa *et al.* (1986), developed from Musashige and Skoog (1962).

Callus culture : The calluses were produced from sections of hypocotyls or cotyledons of seedlings grown in axenic conditions. Explants were placed on 2 media : those of Musashige and Skoog (MS) and Heller. Two hormone combinations were tested : 2.4D 1mg/l + BAP (6.Benzylaminopurine) 1 mg/l and NAA (naphtaleneacetique acide) 2mg/l + BAP 1mg/l. The callus was placed on fresh medium every 3 weeks.

Production of axenic inoculum : The following disinfectants and treatment durations were tried :
NaOCl (12%) for 4 (**T1**), 6(**T2**), 8 min.(**T3**)
NaOCl (8%) for 14min (**T4**) and twice 7 min.(**T5**)
95% ethanol for 10 (**T6**) and 20 sec.(**T7**)
H2O2 55 vol for 10 (**T8**)and 15 min.(**T9**)
"Mercryl" for 15 min.(**T10**)
H_2O_2 55 vol for 10 min + NaOCl (12%) for 1 min.(**T11**)
H_2O_2 55 vol for 10 min + NaOCl (12%) for 2 min.(**T12**)
H_2O_2 55 vol for 10 min + NaOCl (12%) for 3 min.(**T13**)

Results

Production of axenic inoculum : The method of Viranyi and Sziraki (1986), which consisted of sterilizing cotyledons removed from seedlings infected with *P.halstedii* by 2 successive immersions

in 8% sodium hypochlorite was not found satisfactory, as all the tissues were destroyed by this treament. Sterilization treatments were, therefore, carried out on whole seedlings.
The results are presented in the following table :

Treatment	% of cotyledons showing sporulation /20 Rep	% of pure suspensions /20Rep
T1	95	-
T2	100	22
T3	10	-
T4	90	55
T5	85	75
T6	80	-
T7	20	-
T8	100	30
T9	80	-
T10	75	0
T11	100	80
T12	70	78
T13	65	-

With all these treaments, the plants showed some sporulation after 48 h at 18°C in saturated humidity conditions. However, sporulation on cotyledons varied from 100% with the 3 treatments : **T2, T8** and **T11** to only 10% for **T3**. Inoculum was found to be pure (absence of bacteria in 75% of isolations) only with the treatments : **T5, T8** and **T11**. The most efficient protocol thus appears to be this last treatment. However, the easiest to apply was **T5**, since it required only 3 rinsings in distilled water. It gave 80% of cotyledons showing sporulation and 70% pure inoculum. It was this treament that was chosen to produce axenic inoculum.

Plant-parasite co-culture :For this study, the 3 genotypes T9.4, Phoebus and Peredovik were used. Production of healthy callus was possible with all the media tried. However, the Heller medium gave less good results (30 to 60% callogenesis) with cotyledons than with hypocotyls (83 to 100% callogeneis). The hormonal combination NAA+BAP, giving a mean of 87% callus production was slightly better than the combination 2.4D+BAP (84.5% callogenesis).
Infected calluses were produced either by taking explants from downy mildew infected seedlings or by infecting healthy callus. In callus from downy mildew infected seedlings, the development of intercellular hyphae was observed on the edges of callus, with production of haustoria as in normal downy mildew infections of young plants. However, no sporulation was obtained with cultures of up to 2 months, kept at either 18°C or 24°C. Thus, co-cultures of sunflower callus and *P.halstedii* cannot, as yet, be used to produce axenic inoculum.

In vitro resistance test :The embryos were infected when they were changed, after 3 to 5 days, from the first medium to the rooting medium. At this stage, those that showed good development, with opened out, green cotyledons, were immersed in an axenic suspension of zoosporangia, at 18°C for 5h. The embryos were then pricked out on the rooting medium under sterile conditions.
Expression of resistance : *In vitro* culture did not modify reaction to downy mildew, all the genotypes gave the same response as to normal tests on seedlings. Resistant genotypes showed no symptoms, their resistance mechanisms were not affected by *in vitro* culture.

Symptoms : The symptoms observed on susceptible plants were typical of downy mildew : sporulation on cotyledons and chlorosis of leaves. There was no difference in the colonisation of sunflower tissues due to *in vitro* culture. However, the infection rate was always rather low : 37.5% (of 168 plants tested) for race A and 46.7% (170 plants tested) for race B. The levels of infection did not vary between susceptible genotpyes. The test carried out at 24°C gave better infection levels (50%) than those at 18°C (30% infection).

Discussion

These results indicate that it is possible to use rapid fixation by *in vitro* culture to introduce resistance genes into sunflower inbred lines. The axenic suspension of zoosporangia should be produced from disinfected seedlings containing downy mildew (Fig. 1). The resistance test should be carried out when embryos are changed from the first greening medium to the rooting medium (Fig. 2). However, for routine use, it will be necessary to improve production of sterile inoculum, to determine the minimal concentration of zoosporangia to obtain infection without wastage. It will also be necessary to tests a wide range of sunflower genotypes to confirm that responses to an *in vitro* test are always the same as those observed normally.

Acknoledgements

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Fig 1 : Production of axenic inoculum of *Plasmopara halstedii*

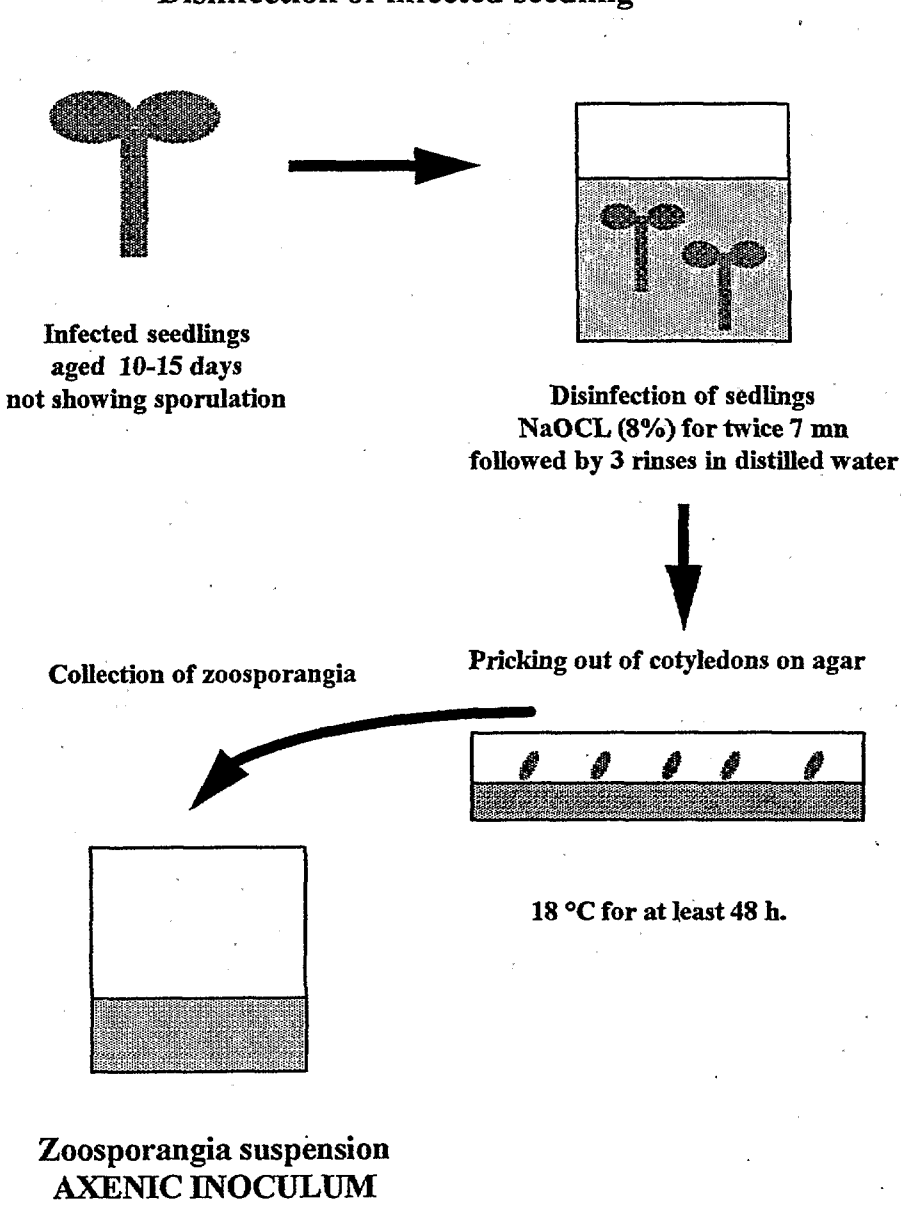
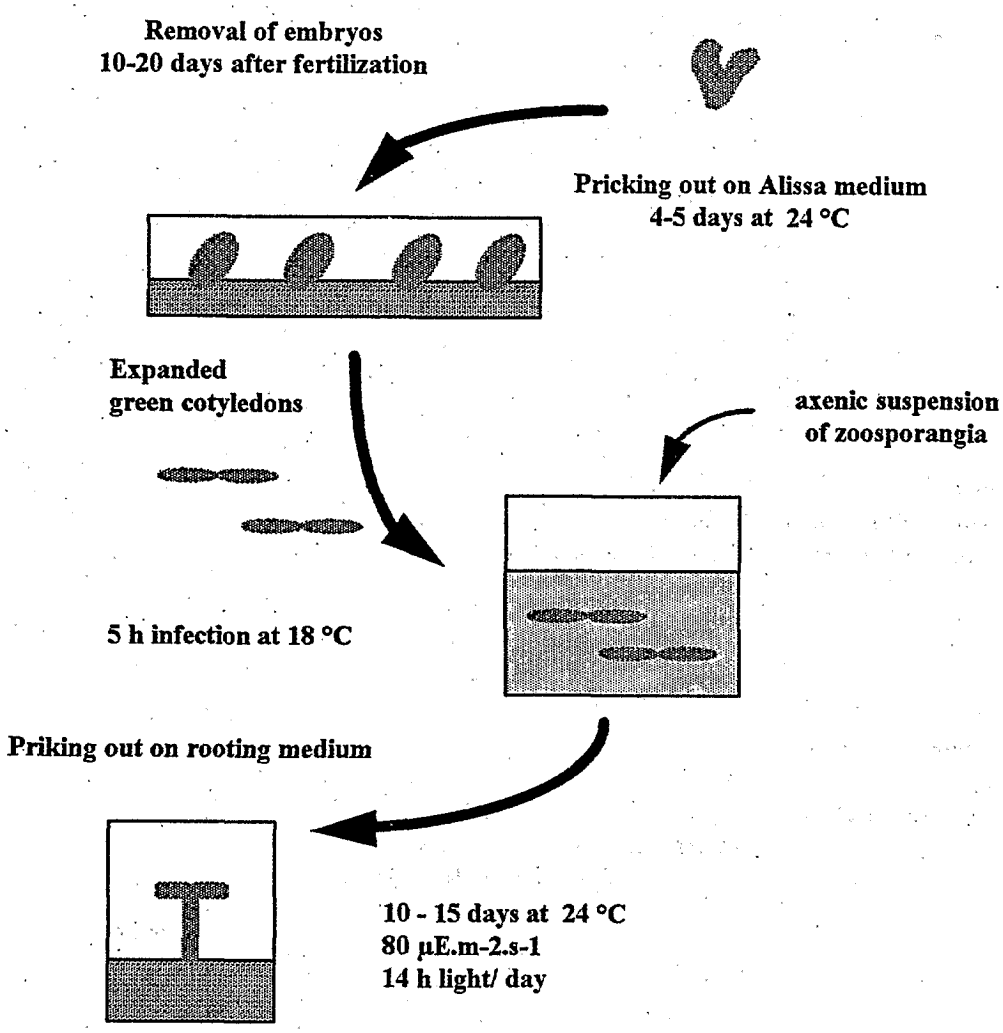


Fig 2 : *In vitro* sunflower downy mildew resistance test



Observations

Susceptible : Sporulation on cotyledons and leaves
Chlorosis of leaves

Resistant : Hypersensitive type reaction