# Introduction of a new specific resistance against *Leptosphaeria maculans* in oilseed rape commercial varieties in France: Monitoring of introduction and control of virulence behaviour to try to avoid resistance break down in a pilot production area in the central part of France

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**Abstract**

Recently new genotypes of oilseed rape were introduced commercially. Their excellent resistance to blackleg (*Leptosphaeria maculans*) is mainly due to a new major resistance gene Rlm7. Such varieties are potentially exposed to a resistance break down risk. That is the reason why we promote specific extension activities and a monitoring in a production area where the risk seems to be particularly high. This area of around 100 Km2 is located in the central region of France between the 2 towns of Issoudun and St Florent sur Cher. In this area 20 to 30 farmer's fields are observed each year. Rlm7 varieties, slightly introduced the previous year, represent 5 % of the total oilseed rape surface in 2005-2006 cropping season. Observations over the seasons include agronomic diagnosis, ascospores releases each autumn, and leaf spots sampling. From sampled leaves, fungus isolates were purified and characterized for virulence profiles. Particular attention was paid to virulence on the Rlm7 reference host. This paper presents results from the two first cropping years. Only a single isolate each year was identified as virulent on Rlm7 reference host, suggesting that the specific resistance would probably stay efficient in the next closed future, even if the virulent inoculum is already present. Nevertheless, such experimental design will continue over the next cropping years, with the aim of been able to detect beginning of fungus population qualitative changes as early as possible.

## Introduction

Stem canker (*Leptosphaeria maculans*) is the most important disease of winter oilseed rape in France and in Europe. Among the possible means able to control the disease, plant genetic resistances is the main spread one. Quantitative resistances and major genes are generally associated in commercial genotypes. Single major gene resistance has been already overcome within a few years of commercial use (Rouxel et al., 2003). In such cases the behaviour of a given cultivar could move rapidly from resistant to susceptible, which is unacceptable for farmers. Actually, several new major genes are identified and tested. Field experiments have shown that such major gene could be quickly overcome (Brun et al 2000). Nevertheless, new commercial lines and hybrids having Rlm7, an efficient new major gene, are coming on the market and start their commercial development. After the first event of Rlm1's break down, applied research bodies are strongly motivated to deploy measures and bio-vigilance to avoid such phenomena. In a recent period CETIOM has promoted an alternance strategy in the use of genotypes based on a fine characterization of both quantitative and major resistances (Pinochet et al 2004). Incorporation of crop residues after harvest to reduce the spread of ascospores inoculum from the stubbles is the second main message actually addressed by CETIOM to farmers. Nevertheless classical communication tools are not sufficient. There is a need to increase credibility and to demonstrate the interest of the recommendations at the production scale. This paper describes a bio-vigilance approach that CETIOM has started to develop in the Center of France in a small production area where the risk of Phoma leaf spot is relatively high and where new Rlm7 varieties are introduced. There are several objectives: (i) to follow in time what is happening in an area of several km² where Blackleg risk is very high: epidemic scenario, symptoms, production impact (ii) To identify criteria for an early detection of a starting break down., (iii)To carry out demonstration site as support of extension and communication activities. (iv) To provide data to feed back modelling activities aiming to predict disease and break down risks and to support integrated protection approaches. This paper presents preliminary results, from the two first years of observation.

## Materials and methods

The working area is located between St Florent sur Cher and Issoudun, over 3 villages at appreciatively 250 kilometres south to Paris. The production area is a square of 10-15Km long. In this area, 20 to 30 Fields were regularly observed each year during the cropping season: emergence, before winter during the second half of November, end of winter, flowering, and beginning of ripening. They represent 400 to 500 Ha of WOSR. Fields and sampling area inside the field were located using a GPS system. Agronomic practices were obtained by questioning farmers. Sampling methods and agronomic observations applied were those recommended in CETIOM's WOSR experimentation guide (Plant density, Plant Fresh weight, Flowering

type, Height, yield components, grain yield). In 2004-05, the main genotypes were: ES Astrid (Rlm9), Aviso (Rlm9), Kosto (Rlm9):, Campala (Rlm9). The Rlm7 line Roxet was only slightly present. In the 20 observed field, only 2 fields were cropped with Roxet. In 2005-06 the market share of Rlm7 restored hybrids reached 5% in the working area. 13 observed Fields were grown with Rlm7 restored hybrids Exagone or Exocet, registered in summer 2004 and summer 2005 in France, respectively. The others fields were grown with Aviso, Es Astrid or Grizzly (Rlm9) which are the leading lines. In 2006-07, we focused our observations on 15 fields cropped with Rlm7 hybrids which performed well in post registration networks and reached 25% market share in the working area. Ascospores trapping are carried out in Rosières, on the east side of the working area, with a dynamic trap from the 1st September to the 30th November 2004, and from the 23rd August to the 30th November in 2005 with daily counts of ascospores under the microscope (Peres et al 1999, West et al 1999). Leaves spots in autumn were recorded from the 15th to the 24th November in the different fields, on 4 samplings of 25 plants. The final disease index G2 was determined from the observation of 8 samplings of 5 plants (Pierre and Regnault 1982, Aubertot et al 2004). 20 leaves per field, with at least one leaf spot per leaf, were collected in November for immediate pathogen isolation. Single-pycnidium isolates were collected from one lesion per affected leaf (West et al 2002). Virulence patterns of the isolated strains will be done according to the procedure described by Balesdent et al 2006. Westar (no Rlm) or Goeland (Rlm9) were used as control genotypes. A PCR procedure was used to distinguish Tox+ and Tox° (*L.biglobosa*) before plant tests (Balesdent et al 1998).

## Results

*Ascospores release*

During three successive years the main ascospores pics were detected only until mid October in 2005 and 2006, but until mid November in 2004. Compared with previous epidemic years like 1999, 2000 or 2001, our working years could be considered as light epidemic years with a low disease pressure. Autumns were rather dry and warm producing plant biomass growth by only late ascospores releases.

*Leaf symptoms*

Leaf symptoms appear late in the season generally 2 or 3 weeks after the first main pic. On classical genotypes having Rlm9 in their genetic background, but no efficient specific resistance, leaves spots could be numerous even with a low biomass, up to 270 spots /m2 in our data. On Rlm7 genotypes, the number of leaf spots is always very low, less than 50 spots /m2 except for two neighbouring fields, sown later with organic amendments before sowing and which produced a particular high vegetative biomass.

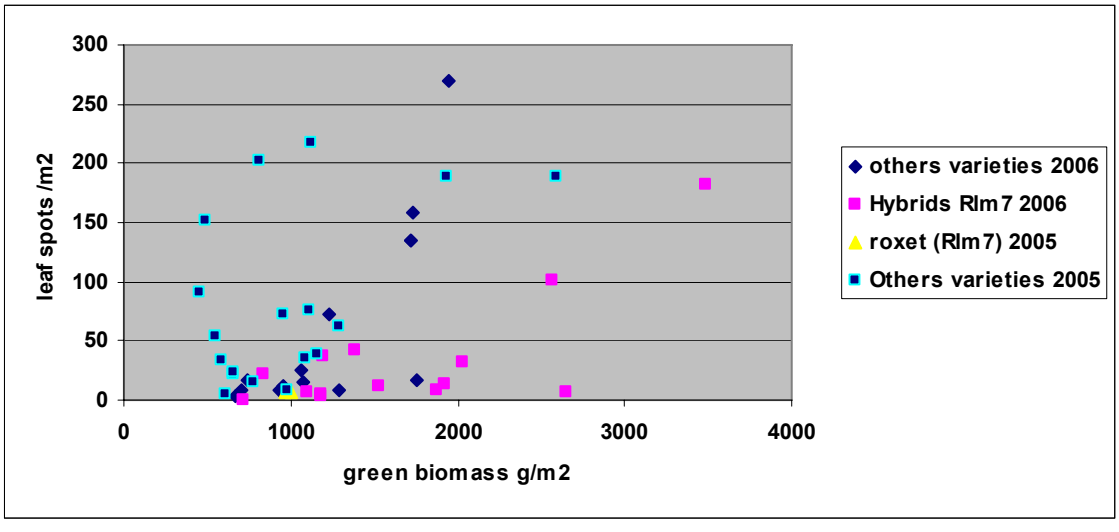


Figure °1: Number of leaf spots per m2 for different types of genotype at the end of November 2004 and 2005

*G2 index*

G2 index were always low, from 0.6 to 4.6 in 2004-05 and from 0.2 to 1.7 in 2005-06. In 2004-05 the two fields of Roxet reached 1.2 and 3.3 respectively. In 2005-06 The Rlm7 hybrids scored only between 0.2 to 0.6 , compared to the classical leading lines which scored between 0.5 to 1.7.

*Tox° / Tox+*

In 2004-05, with only 2 fields with Rlm7 Roxet among 20 observed fields, we only found 2 isolates of L.biglobosa (Tox°), both on Roxet among 181 isolates and 179 identified as *L.maculans* (Tox+).

In 2005-06, among 42 isolates all coming from Rlm7 restored hybrids, *L.biglobosa* isolates represented 17 %.

In 2006-07, with a similar strategy of isolation from Rlm7 restored hybrids, 21 % of the 117 isolates were *L.biglobosa* (Tox °)

*Virulent isolates avrlm7*

*Leptosphaeria maculans* islates (Tox+) were tests on reference hosts for Rlm7 and on a control genotype, Westar (no Rlm) and/or Goëland (Rlm9). For 2004-05, 163 isolates were tested. 162 isolates were avrlm9 / Avrlm7 as expected, and only 1 isolate coming from Roxet was avrlm7/avrlm9. 5 isolates Avrlm7 / avrlm9 were found from Roxet which is supposed with its Rlm7 specific resistance not to allow such result. Nevertheless, many small leaf spots with only a low number of pycnidia were observed from this genotype.

In 2005-06, 35 Williams tests were performed with isolates coming from Rlm7 restored hybrids. 5 isolates were avrlm7 and 30 isolates were Avrlm7. Among the 5 avrlm7, one is sure and the 4 others need to be reconfirmed by a second test.

In 2006-07, William's tests were performed with 108 isolates coming from Rlm7 restored hybrids. 7 avrlm7 isolates were identified but without a second test s needed to be sure, and 101 Avrlm7

For both years we found Avrlm7 isolates on Rlm7 hybrids as we found with Roxet during the first year. Again small leaf spots with a single or very few pycnidia were observed suggesting that the gene for gene interaction is not a perfect barrier in this particular case.

*Rlm7 varieties*

| Season | % of Rlm7 cv in the area | Number of fields with Rlm7 cv in the design | Rlm7 Cultivars |
|---|---|---|---|
| 2004-05 | <1% | 2 / 20 | Roxet |
| 2005-06 | 5% | 14 / 29 | Exagone, Exocet |
| 2006-07 | 25% | 15/15 | Exagone, Exocet, Extend |

In 2005-06, the grain yields from the 14 Rlm7 hybrid fields were from 2.7 to 4.0 t/ha compared to 2.0 to 3.4 t/ha for the leading varieties in the same area. The average difference is 0.4 t more per ha for restored hybrids.

## Discussion

On Rlm7 genotypes which induced a selection pressure, we found avrlm7 isolates. In 2004-05, such result was only coming from Roxet's isolates which suggest, as already demonstrated by Balesdent et al 2006 that the avrlm7 phenotype is already present at a low frequency among *L.maculans* populations. A similar result was found the following seasons with only few avrlm7 isolates for a bigger sampling on Rlm7 genotypes. Considering that the G2 index are also rather low, especially for the Rlm7 restored hybrids cropped near the Roxet field of the first year, we can consider than no signs of a possible break down is actually detected.

Nevertheless such conclusion is strongly limited by the scale of sampling. Looking for a low frequency event, we should have a stronger sampling on both Rlm7 and non Rlm7 genotypes. For such an approach, we are strongly limited by the people available for sampling, for strain isolations, and for large scale Williams tests. Actually no molecular markers are available for such virulence. Recent results from INRA Versailles group suggest that it would be more difficult to find for avrlm7 compared to what has been done for avrlm1, avrlm6 and avrlm4. (INRA Versailles personal communication)

To have an easy and economic way of biovigilance, visual observations of plant leaf spots during autumn on both types of genotypes, those with an efficient specific resistance like Rlm7 and those with no or an overcome specific resistance, could be a possibility. Figure n°1 suggests that such approach is possible. Nevertheles the size of the reception area of disseminated ascopores should be taken into account. The green fresh biomass seems to be an adequate easy estimation of the leaves surface. The indicator could be for Rlm7 genotypes that the number of leaf spots /m2 should not be over 50 spots /m2 for a Fresh Biomass under 2000 g/m2. Such rate has to be improved with more pluriannual data from situations where Break down of the resistance has not yet occured.

Despite such interesting approach, this kind of criteria is not sufficient. Several difficulties have to be solved:

- The first one is that leaf spots number and period of occurrence depend very much of the annual epidemic scenario. Observations has to be undertaken only after a sure contaminating event. This needs spore trapping and counts and to wait 2 or 3 weeks before observations. Observations are secured with a comparative approach with neighbouring fields cropped with a variety without an efficient specific resistance.
- The second difficulty is to be sure of the genetic background of the sampled plant. In oilseed rape fields, it is frequent to have a significative level of volunteers which could be genotypes without an Rlm7 specific resistance. This is a factor of overestimation of number of spots /m2. A plant Rlm7 molecular marker would be appreciate very much to avoid such mistakes
- The third difficulty is to do the observation properly and count effectively the right symptoms. Mistakes could be possible with others pathogens, or with closed species like *Leptosphaeria biglobosa*. Efficient tests are now available to distinguish *L.maculans* and *L.biglobosa* (PCR or PGI marqueurs). We used successfully the PCR test. On varieties known to limit the occurrence of *L.maculans* spots it seems rather logic to find a relatively high level of Tox° spots. Nevertheless the main difficulty and the originality of our work is that we found Avrlm7 spots, often of smaller size, on Rlm7 genotypes, but able to produce a limited number of pycnidia. It is always difficult, even for a trained technician, to distinguish classical and such original leaf spots. A collection of numerical pictures with a sure correspondence between the symptom and the isolate identity is actually developed to produce a training CD.

- The fourth difficulty is the phenotype Avrlm7 it self. An isolate is said Avrlm7 if it is avirulent on a cotyledon test on the reference cultivar, and a genotype is qualified as Rlm7 after the same kind of tests against reference strains. But we do not know levels of variability for the Rlm7 specific resistance gene among genotypes called Rlm7 and among the fungus population characterized as Avrlm7. The expression of the gene for gene interaction could also be depend from several factors like plant growth stage, or environmental conditions. Recently, Huang et al (2006) show that the Rlm6 /avrlm6 interaction is dependant from the temperature and the wetness duration

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## References

Aubertot JN., Schott JJ, Penaud A., Brun H, Doré T. 2004 Methods for sampling and assessment in relation to the spatial pattern of phoma stem canker (*Leptosphaeria maculans*) in oilseed rape. European Journal of Plant Pathology 110:183-192.

Balesdent M.H., Jedrysczka M., Jain L., Mendes-Pereira E., bertrandy J., Rouxel T.1998. Conidia as a substrate for internal transcribed spacer-based PCR identification of members of the *Leptosphaeria maculans* species complex. *Phytopathology*, 88: 1210 - 1217.

Balesdent MH, Louvard K, Pinochet X, Rouxel T 2006 A large-scale survey of races of *Leptosphaeria maculans* ocurring on oilseed rape in France. European Journal of Plant Pathology. 114:53-65.

Brun H, Levivier S, Ruer D, Somda I, Chèvre AM and Renard M (2000) A field method for Evaluating the potential durability of new resistance sources: application to the *Leptosphaeria maculans/Brassica napus* pathosystem. Phytopathology 90: 961-966.

CETIOM novembre 2005: Guide de l'expérimentateur colza. Edition CETIOM ISBN:2-908645-64-5.

Huang YJ, N.Evans, ZQ Li, M.Eckert, AM Chevre, M.Renard, B.Fitt, 2006 Temperature and leaf wetness duration affect phenotypique expression of Rlm6-mediated resistance to *Leptosphaeria maculans* in *Brassica napus* New phytologist 170: 129-141

Peres A, Poisson B, Penaud A, Jain L, Pilorge E 1999, Leptosphaeria maculans (Phoma lingam): A summary of three years of epidemiological studies (1995, 1996, and 1997). Proceedings of the 10th International Rapeseed Congress, Canberra, Australia.

Pierre JG et Regnault Y 1982 Contribution à la mise au point d'une méthode de plein champ destinée à mesurer la sensibilité des variétés de colza au Phoma. Informations Techniques du CETIOM 81:3-18.

Pinochet X, Balesdent MH, Salvi F, Mestries E, Rouxel T 2004 Identification of specific plant resistance factors to Phoma (Leptosphaeria maculans) among winter oilseed rape varieties: interest for variety testing and for the promotion of a first step of a durable management of resistances. International Organisation for Biological Control wprs Bulletin 27: 101-108

Rouxel T., Penaud A., Pinochet X., Brun H., Gout L., Delourme R., Schmit J., Balesdent M-H. 2003: A ten-year survey of populations of *Leptosphaeria maculans* in France indicates a rapid adaptation towards the *Rlm1* resistance gene of oilseed rape. European Journal of Plant Pathology 109: 871-881.

West JS, Biddulph JE, Fitt BDL, Gladders P 1999 Epidemiology of *Leptosphaeria maculans* in relation to forecasting stem canker severity on winter oilseed rape in the UK. Annals of Applied Biology. 135: 535-546

West JS, Balesdent MH, Rouxel T, Narcy JP, Huang YJ, Roux J, Steed JM, Fitt BDL, Schmit J 2002.Colonization of winter oilseed rape tissues by A/Tox+ and B/Tox° Leptosphearia maculans (Phoma stem canker) in France and England. Plant Pathology 51:311-321.