PARTIAL RESULTS REGARDING IDENTIFICATION OF A RESISTANCE SOURCE OF OILSEED RAPE AT *Verticillium longisporum* PATHOGEN

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Abstract

Brassica napus is consider to be one of world's important oil crops which provides not only the edible oil for human consumption but also the protein rich feed for animals and raw materials for industrial processes such as biodiesel production. The aim of our study was to indentify some new resistant sources of oilseed rape with resistance to *Verticillium longisporum* disease. For this as biological material we used of 65 oilseeds rape cultivars. To obtain the phenotypic data regarding the *V. longisporum* resistance, all cultivars were artificial inoculated with the pathogen in laboratory. After the artificial infection, it was observed a large variation of resistance to *V. longisporum* among the tested cultivars. We obtained 15 cultivars which showed a higher resistance to the pathogen than the control variant Express which is tolerant to the disease. For the molecular studies, we used 50 SSR markers which were chosen from previous studies. Using the genotypic data obtained with the SSR analysis and the phenotypic data represented by the AUDPC values resulted after the artificial infection of the each cultivar, we identified a QTL for resistance to *V. longisporum*. The QTL was localized on the first chromosome (LG group 1) with a LOD valued of 3,4 and a phenotypic variation (R^2) of 11,4%. The results from this study are the first step in the investigation of the genetic basis of currently available resistance sources.

Key words: oil seed rape, *Verticillium longisporum*, resistance

Oilseed rape (*Brassica napus* L.) is a relative young species species which appeared a few hundred years ago through a spontaneous interspecific hybridization between cabbage (*Brassica oleracea* L.) and turnip rape (*Brassica rapa* L.) (Rygulla et al., 2007).

Due to the high content of oil seeds, rapeseed has become one of the most important oilseed plant, being a valuable source of oil and protein used in human food, animal feed and the production of biofuel.

For this reason, in Romania the cultivated areas with this crop started to increase in the last period and it is expected to increase more in the future. As a consequence of this, the farmers will start to deal with more diseases, weeds and pests in the culture.

Verticillium wilt caused by the fungal pathogen *Verticillium longisporum* is one of the most important pathogens of oilseed rape (*Brassica napus*) besides blackleg and stem root (Enynck et al., 2007). The disease is especially prominent in northern Germany and Sweden, but it has also been reported to occur in Poland, France, southern Russia, and Ukraine. Because until now there are no approved chemicals for this disease, the use of resistant cultivars within an integrated disease management approach is the most promising strategy for controlling *Verticillium* wilt. Currently available commercial varieties are generally susceptible or exhibit only slight tolerance to *V. longisporum* (Dunker S., 2006).

The genetic base of the molecular mechanism involved in the resistance of the oilseed rape cultivars against *V.longisporum* is poorly understood. The most promising sources of resistance from *B. oleracea* were crossed with winter turnip rape (*B. rapa*) to produce resynthesized rapeseed lines for crossing with advanced oilseed rape breeding material (Happstadius et al., 2003).

New sources of resistance have also been identified in *B. oleraceea* and in *B. rapa* (Rygulla et al., 2007a). Four different chromosome regions with quantitative trait loci (QTL) have been identified, with the two major QTL regions being on the C-genome. The resistance is therefore likely to have originated from *B.oleracea* (Rygulla *et al.,* 2007b). Mapping populations have been produced and molecular markers need to be identified to assist the selection of resistant loci (Rygulla et al., 2007a,b).

The aim of this study was to indentify QTL for resistance against *V. longisporum* in order to

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offer information for the genetics of new, resistant *B. napus* breeding lines.

MATERIAL AND METHOD

Plant material

The plant material for this study comprised 65 genotypes of rapeseed cultivars proceeded from the Centre for Genetic Resources Netherlands. The plants were sown in the field in order to collect the fresh tissue material for DNA extraction. The details about the oilseed rape cultivars are shown in *table 1*.

DNA extraction and SSR analysis

Leaf samples were taken from all plants of each cultivar, bulked and immediately frozen in liquid nitrogen. DNA was extracted using the CTAB procedure, modified according to Doyle and Doyle (1990). DNA content was measured using a Nano Drop spectrophotometer. Based on these data DNA was diluted to a concentration of 20 ng/ µl for SSRs.

For the SSR analysis we selected 50 SSR primers which were used previously used in several studies at Brassica napus (Radoev et al., 2008; Rygulla et al., 2008; Hasan et al., 2004).The PCR reactions were carried out in 10 µl containing 20 ng of DNA template, 0.75 pmol of each primer, 0.2 mM dNTP mix, 1 mM MgCl2, 10XPCR reaction buffer and 5 unit of Taq DNA polymerase (Qiagen).

Amplifications were performed using a standard amplification cycle in a GeneAmp PCR System 9700 thermal cycler, and SSR polymorphisms were separated and visualized using a LI-COR GeneReadir 4200 (MWG Biotech, Ebersberg). To reduce primer labeling costs PCR products were labeled with the M13-tailing technique. In this method the fluorescently labeled universal M13 primer 5'-AGGGTTTTCCCAGTCACGACGTT- 3' is added to the PCR reaction, and the forward primer of each SSR is appended with the sequence 5'-TTTCCCAGTCACGACGTT-3'.

Table 1

The 130 oilseed cultivars used in this study

Nr. Crt.	*Name of accession*	*Country of origin*	Nr. Crt.	*Name of accession*	*Country of origin*
1.	Libritta	Germany	**34.**	Mirander	Germany
2.	Skriverskii	Lithuania	**35.**	Niederarnbacher	Germany
3.	B. napus group 1	Ukraine	**36.**	Norli	Germany
4.	Kievskii 216	Ukraine	**37.**	Octavia	-
5.	Kievskii 18	Ukraine	**38.**	Olimpiade	Italy
6.	Kombi	Ukraine	**39.**	Olymp	Germany
7.	SKR. II Kormovoi	Lithuania	**40.**	Panter	Sweden
8.	Uspekh	Ukraine	**41.**	Perle	Germany
9.	Blagodatnyi	Ukraine	**42.**	Andol	France
10.	Fedorovskii	Ukraine	**43.**	Arabella	Germany
11.	Snityskii	Ukraine	**44.**	Bienvenu	France
12.	Diana	Germany	**45.**	Brilland	Poland
13.	Ksaverovskii	Ukraine	**46.**	Bristol	France
14.	Kodakskii	Ukraine	**47.**	Buko	-
15.	Lictor	Germany	**48.**	Capricorn	Great Britain
16.	Liglandor	Germany	**49.**	Cobra	Germany
17.	Ligora	Germany	**50.**	Collo	Germany
18.	Lindora	Germany	**51.**	Planet	Germany
19.	Lingot	France	**52.**	Prominj	Russia
20.	Link	-	**53.**	Ridana	Germany
21.	Liquanta	Germany	**54.**	Samourai	France
22.	Lirabon	Germany	**55.**	Score	Great Britain
23.	Lirajet	Germany	**56.**	Silesia	Czechoslovakia
24.	Lirakotta	Germany	**57.**	Silvia	Germany
25.	Lirama	Germany	**58.**	Sollux	Germany
26.	Lirastern	Germany	**59.**	Susana	Germany
27.	Lirektor	Germany	**60.**	Tamara	Germany
28.	Liropa	Germany	**61.**	Tapidor	France
29.	Madora	Germany	**62.**	Tor	Sweden
30.	Maras	Poland	**63.**	Veronika	Germany
31.	Marens	France	**64.**	B. napus group 2	Ukraine
32.	Marex	Germany	**65.**	B. napus group 3	Moldavian Republic
33.	Matador	Sweden			

The amplification was performed after a touch-down PCR cycle was modified from the procedure described by Xu et al. (2005) as follows: An initial denaturation was performed at 95°C for 2 min, followed by five cycles of denaturation for 45 s at 95°C, annealing for 5 min beginning at 68°C and decreasing by 2°C in each subsequent cycle, and extension for 1 min at 72°C. Then five cycles were performed with 45 s denaturation at 95°C, 1 min annealing beginning at 58°C and decreasing 2°C in each subsequent cycle, and 1 min of extension at 72°C. The PCR was then completed with an additional 27 cycles of 45 s denaturation at 94°C, 2 min. of annealing at 47°C, and 30 s of extension at 72°C, with a final extension at 72°C for 10 min.

The visualization of the amplified fragments was made using Saga generation software version1. Each primer was scored manual, for the presence of the band using ‘1“and 0 were the band was absent. For the QTL identification we used Join Map software, version 3.

Resistant tests for V. longisporum

The resistance test were performed with V longisporum isolate VL 43 which was provided by division of Plant Pathology and Plant Protection, Gottingen, Germany. For long-term storage we prepared a conidial suspension in a concentration of 1–3 × 106 conidia mL–1 in Czapek Dox medium supplemented with 25% glycerol at –80°C. The inoculum suspension was made by adding 500 µl spore stock solution in 250 ml PDB medium. The cultures were subsequently incubated 7 days at 230C on a rotary shaker. After 7 days the culture was filtered and using a haematocytometer we determined the spore concentration. For the inoculation we diluted the solution at 1x106 spore mL-1.

The winter oilseed rape varieties Express (less susceptible) and Falcon (highly susceptible) were used as reference control in our experiments. The seeds were two times surface sterilized by immersion in 70% ethanol for 2 minutes. After the sterilization, the seeds were washed with tap water and then sowed in silica sand. After 10 days, the roots of the plans were carefully washed from the sand. Inoculations were performed by cutting 2 cm from the roots and hold them for 30 min in the spore suspension. Plants from the controls were also cut and hold 30 min in tap water. For each cultivar we used 10 plants inoculated and 10 controls. Plantlets were transferred after inoculation in pots into a mixture of sand, peat and compost (1: 1:2) and grown in a climatic chamber at 230C with a light/dark cycle of 1/10. Every week we take the disease scores using an assessment key with nine classes as described by Eynck et al 2007 (table 2). Because the disease produced by Verticillium longisporum reduces the plant growth, the plant height was measured at 28 days after inoculation.

For each accession, area under disease progress curve (AUDPC) was calculated from the disease severity values.

Table 2

Assessment key for scoring disease severity

Score	Symptom development
1	No symptoms
2	Slight symptoms on the oldest leaves (yellowing, black veins)
3	Slight symptoms on the next younger leaves
4	About 50% of the leaves show symptoms
5	More than 50% of the leaves show symptoms
6	Up to 50%of the leaves are dead
7	More than 50% of the leaves are dead
8	Only apical meristem is still alive
9	The plant is dead

RESULTS AND DISCUSIONS

In order to identify the resistant cultivars to *V. longisporum*, the oilseed rape genotypes were artificially inoculated with the pathogen in the green house. After the artificial infection, typical symptoms like asymmetric yellowing of leaves and early stunting were observed. Plants of the control variant were also scored in order take into account the unspecific symptoms occurring during the natural ageing process which varied between the cultivars. After the observations that were made, it was observed a large variation of resistance to *V.longisporum* among the tested cultivars. Resistance responses of the tested cultivars along with the oilseed rape controls “Express” and “Falcon” measured by AUDPC values are shown in *figure 1*.

As it can be observed in figure 2, a number of 15 cultivars showed a high level of resistance, with the AUDPC values smaller than the cultivar Express.The most resistant cultivar to the disease proved to be the cultivar “*Brassica napus* group 1”, with the smallest value of the AUDPC of 0.10. The next cultivars that proved a high level of resistance recorded AUDPC values between 0.34 - 0.64 and proved to be some promising sources of resistance in our further studies. In order to identify the genomic segments that are responsible for some qualitative characters, the QTL analysis was made.

The QTL identification was made using the Join map software version 3 using a LOD threshold of 3.0. The QTL result are presented in *figure 2.*

Using the genotypic data obtained with the

SSR analysis and the phenotypic data represented by the AUDPC values resulted after the artificial infection of the each cultivar, we identified a QTL for resistance to *V. longisporum*. The QTL was localized on the first chromosome (LG group 1) with a LOD valued of 3,4 and a phenotypic variation (R^2) of 11,4%.

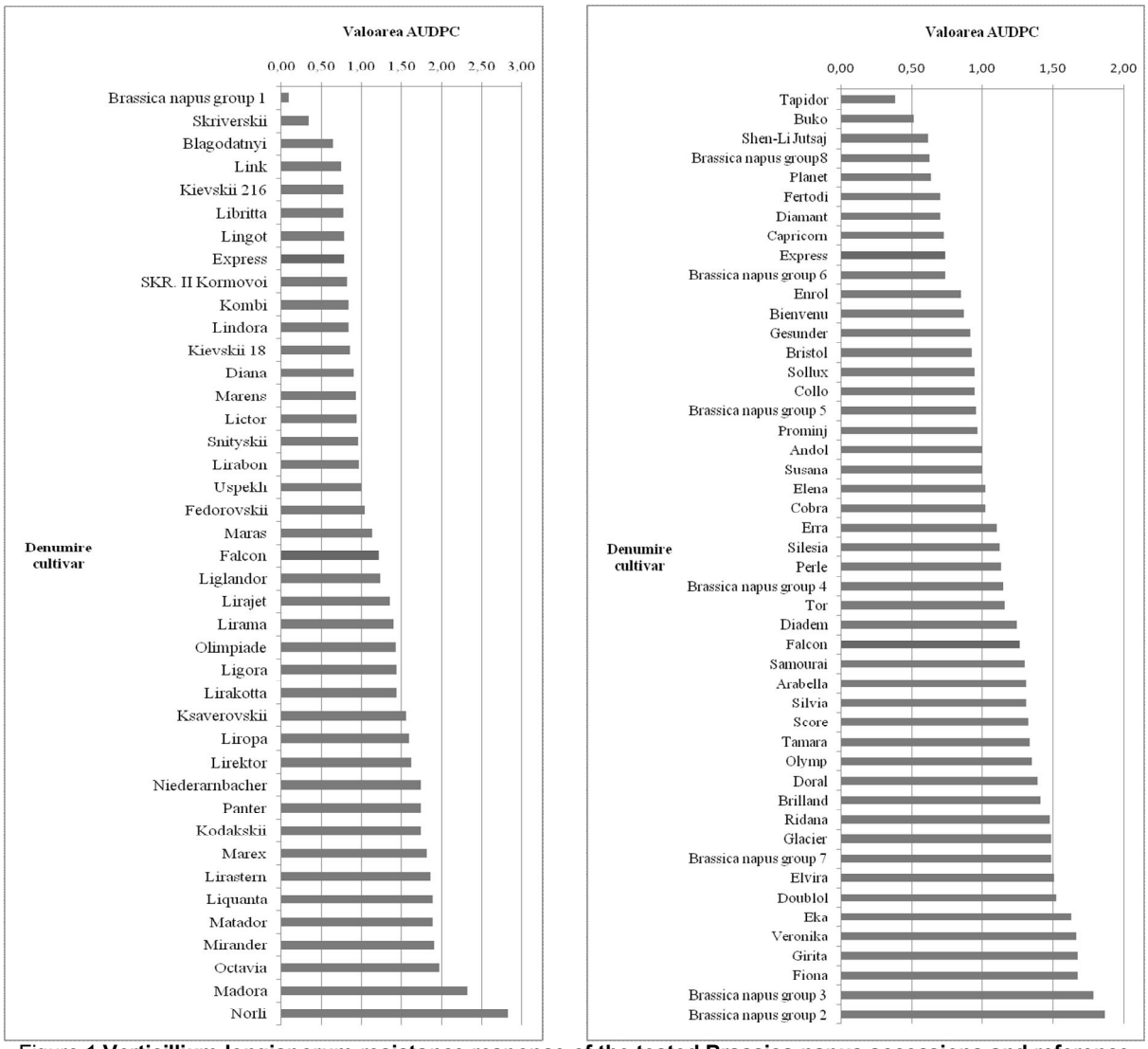

Figure 1 **Verticillium longisporum resistance response of the tested Brassica napus accessions and reference cultivars Express and Falcon (red) measured by area under disease curve (AUDPC). Colums and whiskers present mean values and standard errors from 10 infected plants of each accession**

The 65 oilseed rape cultivars tested in this study showed a differential variation in resistance to *Verticillium longisporum* compared with "Express"(tolerant) and "Falcon"(susceptible) used as controls in this experiments. After the artificial infection with *V. longisporum*, from all the tested cultivars 16% proved to be more resistant than "Ëxpress"and 30 % proved to be more sensible than "Falcon". Similar results had been obtained by Happstadius et al. (2003) in a study where they tested some *B. rapa* cultivars to *V. longisporum*.

Sources of resistance to *Verticillium longisporum* had been identified previously at some *B. oleracea* and *B. carinata* species (Debode J. et al., 2005; Zeise K. et al, 1992) these being already used in synthetic hybridizations for transferring the resistance genes at *Brassica napus* (Rygulla W. et al, 2007).

Based on the phenotypic data obtained after the artificial infection and molecular data obtained after SSR amplification we identified a QTL for resistance to *V. longisporum*. The SSR markers used in this study were chosen from different genetic maps made at *Brassica napus* (Radoev M. et al., 2008; Basunanda P. et al, 2008; Rygulla W. et al, 2007).

From all 51 SSR markers that were used for QTL calculation, we identified 18 markers that

showed phenotypic variations bigger that 5% for the AUDPC values calculated after the artificial infection. Some of these markers were used in previous studied for QTL identification for the resistance to *V. longisporum* in synthetic *Brassica napus* populations (Rygulla W. et al, 2008).

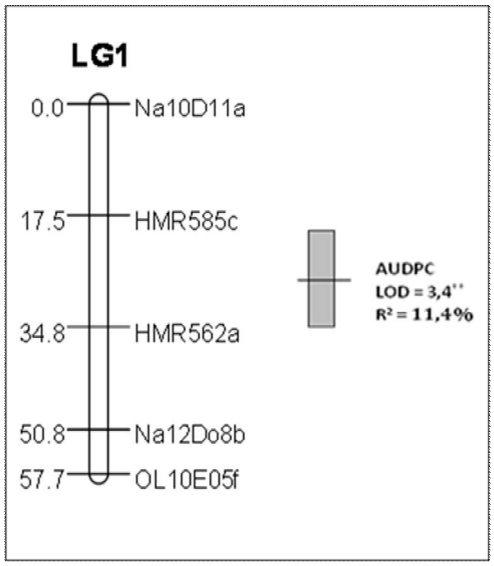

Figure 2 **The indentified QTL for *V. longisporum* resistance**

In this study, the QTL for resistance to *V. longisporum* was identified on C1 chromosome in a diverse genetically population. This result is similar with those obtained by Obermeier et al. (2012) which also identified a QTL for verticillium resistance in segregating DH *Brassica napus* population. Because in this case, the QTL was identified in a diverse population it can be assumed that on this chromosome may be localized one of the genes that is controlling the resistance to *V. longisporum* at Brassica. This can be a valuable information for further studies in the breeding programs for obtaining new commercial hybrids resistant to *Verticillium longisporum*.

The results from this study are the first step in the investigation of the genetic basis of currently available resistance sources.

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