HELIA, **34**, Nr. 55, p.p. 91-100, (2011) UDC 633.854.78:631.523:633.494
DOI: 10.2298/HEL1155091M

DNA POLYMORPHISM OF WILD SUNFLOWER ACCESSIONS HIGHLY SUSCEPTIBLE OR HIGHLY TOLERANT TO WHITE ROT ON STALK

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Received: November 15, 2011
Accepted: December 10, 2011

SUMMARY

DNA polymorphism in accessions of *Helianthus* mollis, *Helianthus rigidus* and *Helianthus tuberosus* differing in level of resistance to stem white rot caused by *Sclerotinia* was evaluated in reactions with two RAPD primers, C04 and C15, selected for their potential linkage with resistance to *Sclerotinia*. In total, 36 fragments using both primers were generated. All fragments generated by C04 were polymorphic, while 5 fragments out of 11 generated by C15 were monomorphic. Two major clusters were identified on the phenogram generated by UPGMA analysis, one comprising plants of *H. mollis* 1298, and the other where all other accessions were grouped. In order to check and statistically confirm any significant association of specific fragments either with resistance or susceptibility, contingency coefficient test was done. Out of 36 fragments generated, 26 were significantly associated with resistance/susceptibility. Fragment C04-950 bp was found to be significantly associated to susceptibility, as it was generated only in susceptible accessions, while fragment C04-1200 bp was found to be associated with the resistance, as it only occurred in *H. mollis* accessions. The selected markers should be checked and results verified in further studies including higher number of wild sunflower accessions differing in their reaction to stem white rot caused by *Sclerotinia.*

Key words: ***Helianthus*, *Sclerotinia sclerotiorum*, tolerance, RAPD**

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important sources of vegetable oil in the world, but the traditional breeding programs still have difficulties solving some of the problems in sunflower production, like susceptibility to dis-

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eases. *Sclerotinia sclerotiorum* (Lib.) de Bary is causing agent of white rot, one of the major sunflower diseases in humid weather conditions (Maširević and Gulya, 1992). There are no suitable cultivation or agrochemical methods for control of *Sclerotinia*, so the breeding for resistance is the only way to enable stable sunflower production in the area affected with this disease. Different forms of this fungus attack many plant parts: root, stem, capitulum and bud, and the reaction to the pathogen is under polygenic control. It means that plant organs can have quite different levels of resistance and express independent reactions during the infection (Robert *et al.*, 1987; Castaño *et al.*, 1992; Roenicke *et al.*, 2004), so each form of attack on the plant must be considered as different disease (Mestries *et al.*, 1996).

The complexity of the resistance mechanisms significantly complicates development of resistant sunflower hybrids and prevents development of fully tolerant or resistant genotypes (Bazzalo *et al.*, 1991). Wild *Helianthus* species constitute a useful source of genes responsible for resistance to pathogens (Georgieva-Todorova, 1976), including *Sclerotinia*. Škorić and Rajčan (1992) and Henn *et al.* (1997) found the highest level of resistance to *Sclerotinia* stem infection in *Helianthus maximiliani* accessions, while Vasić *et al.* (2002) detected the highest level of resistance to the same infection in accessions of *Helianthus mollis*.

The random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1993) marker system has been used in many different applications in crops breeding and genetic research. In sunflower, RAPD markers have been used for evaluation of genetic diversity in wild species and cultivated genotypes (Vasić *et al.*, 2003; Iqbal *et al.*, 2010; Ribeiro, 2010), identification of progenies from interspecific crosses (Taški-Ajduković *et al.*, 2006, Popović *et al.*, 2008), as well as for genome mapping and tagging tolerance genes to rust (Lawson *et al.*, 1998) and broomrape (Lu *et al.*, 2000). They have been used in identification of white mould resistant genotypes in garlic (Nabulsi *et al.*, 2001) and snap bean (Chung *et al.*, 2008).

The aim of this study was to investigate DNA polymorphism in accessions of *Helianthus mollis* Lambert, *Helianthus rigidus* (Cass.) and *Helianthus tuberosus* L. differing in level of resistance to stem white rot caused by *Sclerotinia*, by using selected RAPD primers.

MATERIAL AND METHODS

Accessions of *Helianthus mollis, Helianthus rigidus* and *Helianthus tuberosus*, originating from the wild sunflower species collection of Institute of Field and Vegetable Crops, Novi Sad, Serbia, were used in the study (Table 1).

Table 1: Wild sunflower accessions used in the study and their tolerance to mid-stalk white rot

Accession name[1]	Genebank	Synonym	Country	Tolerance (%)
MOL 1298	SRBIFVCNS	PI 468759	USA	100
MOL X	SRBIFVCNS	-	Unknown	100
RIG 1844	SRBIFVCNS	PI 494613	USA	0
TUB 6	SRBIFVCNS	-	Montenegro	0

[1]MOL - *H. mollis*, RIG - *H. rigidus*, TUB - *H. tuberosus*

The accessions were pre-screened for their tolerance to mid-stalk white rot following the protocol of Vasić *et al*. (2002).

DNA isolation from leaf samples of nine individual plants per accession was done following a CTAB protocol by Somma (2004). Concentration of obtained DNA was measured by visualization on 1% agarose gels in 0.5×TBE buffer with ethidium bromide (0.5 g/ml) added to the gel. The λ phage DNA concentrations of 10 ng, 20 ng and 50 ng were used as standards. Images were taken under UV light.

The quality of the isolated DNA was evaluated by PCR reaction with universal primers U_1 (5'-TCT GCC CTA TCA ACT TTC GAT GCT A)- 3' and U_2 (5'-AAT TTG CGC GCC TGC TGC CTT CCT T-3') specific for 18S rRNK, part of the small ribosomal subunit, following the amplification program: denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 25s, 60°C for 30 s and 68°C for 45 s, with final extension at 68°C for 10 min. PCR products were separated on 2% agarose gels. Marker FastRuler^{TM}, Low Range, ready to use (Fermentas) was used as size reference.

DNA polymorphism was evaluated in reactions with two RAPD primers, C04 (5'-CCGCATCTAC-3') and C15 (5'-GACGGATCAG-3') (Sossey-Alaoui *et al.*, 1998), selected for their potential linkage with resistance to *Sclerotinia* (Vasić, 2003). PCR amplification was done in 25 μl reaction volume containing 2.5 μl of reaction buffer (Fermentas); 1.5 mM $MgCl_2$, 0.2 mM dNTP; 0.5 μM primers, 2 unit Taq polymerase (Fermentas) and approx. 100 ng DNA were used.

Amplifications were carried out in a Mastercycler ep gradient S termocycler (Eppendorf) with the following program: denaturation at 94°C for 4 min followed by 40 cycles of 94°C for 2 min, 36°C for 1 min and 72°C for 2 min, with final extension at 72°C for 10 min. PCR products were visualized on 1.7% agarose gel. A 50bp DNA Step ladder (Sigma) was used as size reference.

Each fragment that was amplified using RAPD primers was treated as binary unit character and scored "0" for absence and "1" for presence. An unweighted pair group arithmetic mean method (UPGMA) cluster analysis was performed, using average linkage method. Association between RAPD markers and tolerance to the *Sclerotinia* was measured by contingency coefficient. The statistical significance of the association was evaluated by independence test. Statistical analysis was carried out using STATISTICA 10 (StatSoft, 2011).

RESULTS AND DISCUSSION

After isolation, the DNAs were present on the gel as a high molecular weight band in all samples (Figure 1). For additional quality testing, isolated DNAs were used as template in amplifications with universal primers. In all samples the size of obtained products was 150 bp (Figure 2) indicating that the quality of isolated DNA is suitable for RAPD-PCR analysis. The results confirmed that the CTAB protocol (Somma, 2004) is an appropriate for DNA isolation from leaves of wild *Helianthus*

species, rich in polysaccharides and polyphenoles, as it enabled us to get satisfactory quantities of good quality DNA. Compared to other methods most frequently used for DNA isolation in sunflower, such as modified CTAB protocol (Permingeat *et al.*, 1998) and method described by Gentzbittel *et al.* (1994), this method is rather simple and less time consuming, as it takes only 3 to 4 hours.

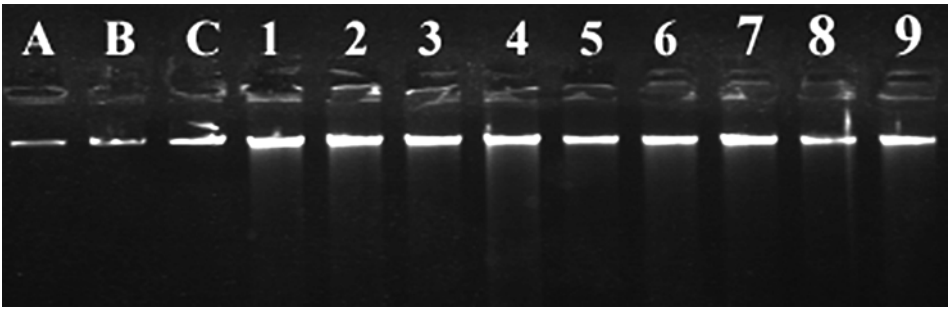

Figure 1. DNA from leaves of individual plants of accession H. mollis x (standard DNA: A-10 ng, B-20 ng, C-50 ng)

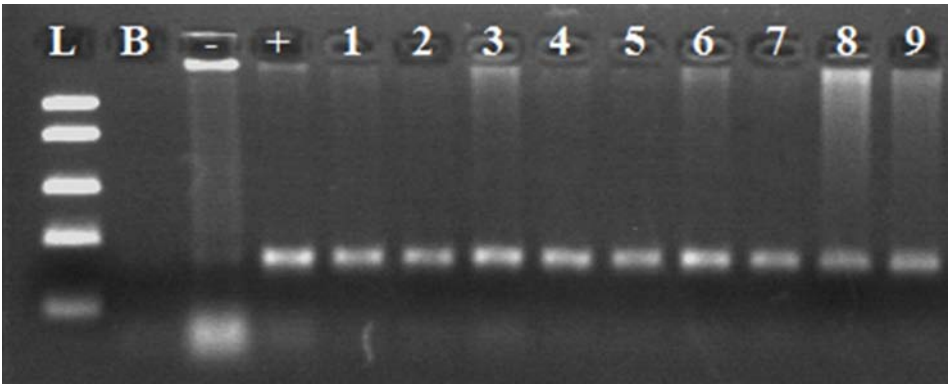

Figure 2. Agarose gel electrophoresis of PCR products obtained using the universal primers (L-DNA ladder, B-blank, - bacterial DNK, + positive control, 1-9 H. mollis x)

DNA polymorphism of selected accessions, with different resistance to white rot on stalk, was analyzed by RAPD, using primers C04 and C15. These primers were selected for their ability to amplify *H. maximiliani* 1631 fragments in somatic embryos regenerating *in vitro* on selective medium with oxalic acid (Vasić, 2003). It indicates that these primers are linked to the inserted *H. maximiliani* region, which might have role in regulation of *Sclerotinia* resistance.

In total, 36 fragments using both primers were generated. All fragments generated by C04 were polymorphic, while 5 fragments out of 11 generated by C15 were monomorphic.

The phenogram generated by UPGMA analysis is shown in Figure 3. Two major clusters were identified, one comprising plants of *H. mollis* 1298, and the other

where all other accessions were grouped. In the latter group, plants of *H. mollis* x formed separate sub-cluster, while plants *H. rigidus* and *H. tuberosus* were relatively similar and were grouped within the same sub-cluster. Higher variability among the plants of the same accession was observed only in *H. mollis* x, while other accessions were rather uniform. Similar to the results obtained by other authors who used RAPD markers in wild sunflower species (Lawson *et al.*, 1994; Sossey-Alaoui *et al.*, 1998; Vasić *et al.*, 2003; Ribeiro *et al.*, 2010) our results corresponded mostly to the phylogenetic relations within the genus *Helianthus*. However, there was significant dissimilarity between two accessions of *H. mollis*, where accession MOL X was found to be more similar to accessions of plants *H. rigidus* and *H. tuberosus* than to the other *H. mollis* accession.

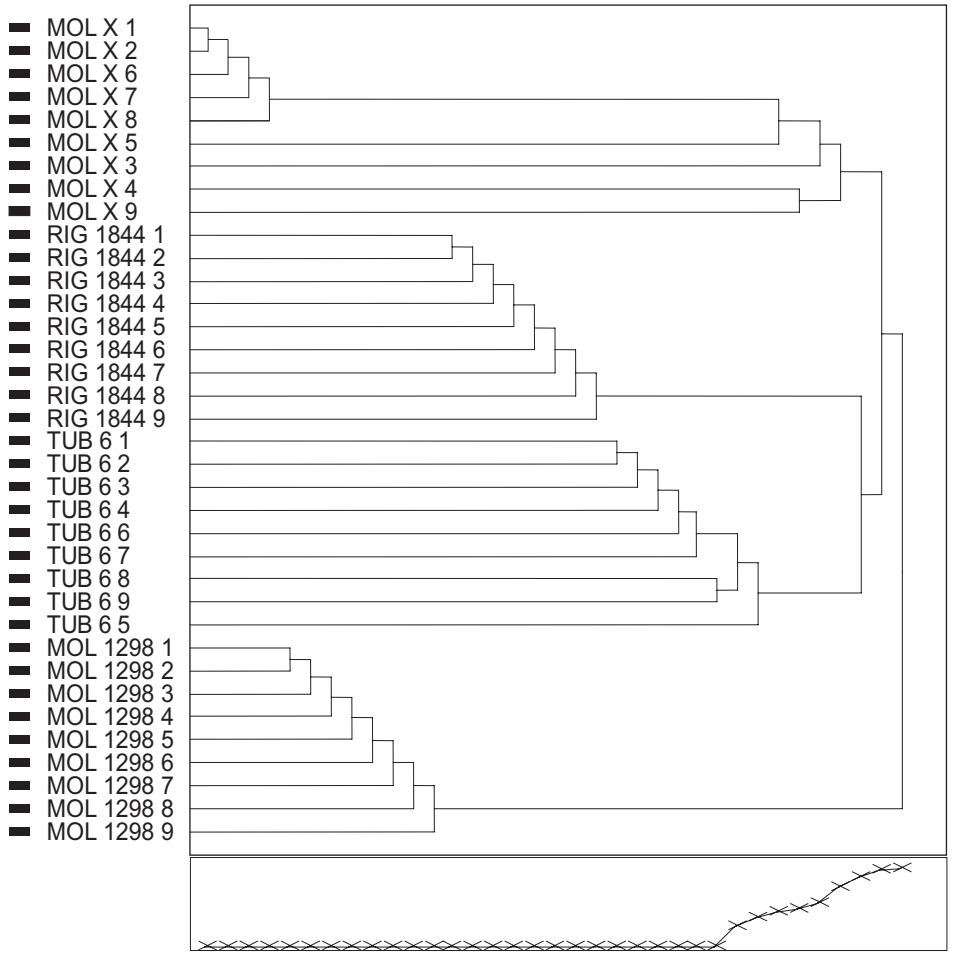

Figure 3. Dendrogram showing relationship among different accessions of tested Helianthus species based on RAPD markers using UPGMA algorithm and average distances

After amplification with primer C04 it could be detected that there was polymorphism in accession *H. mollis* x (Figure 4).

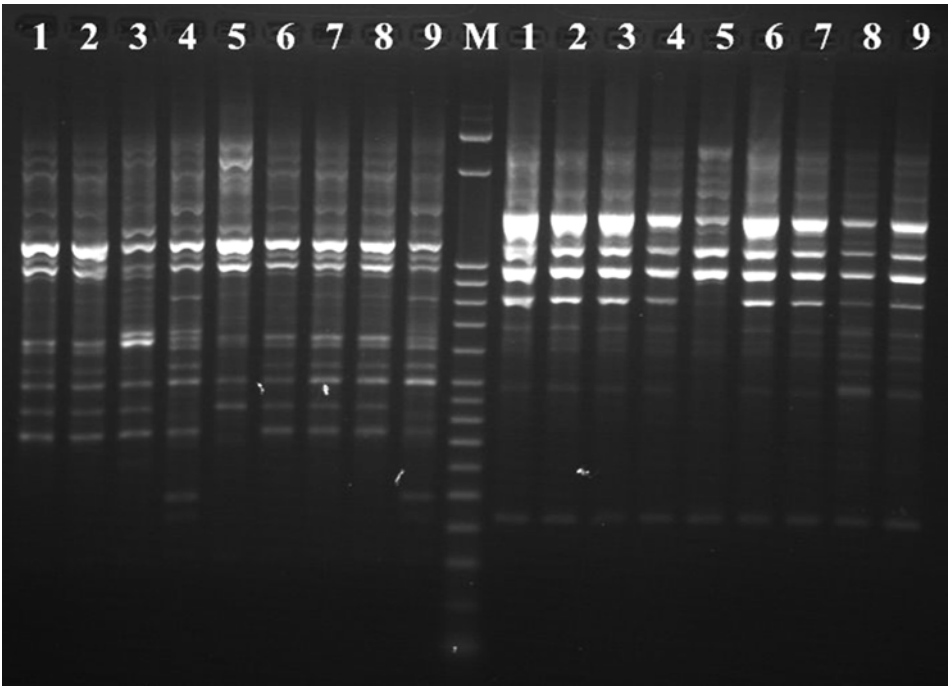

Figure 4. RAPD profile obtained with C04 primer
(1-9 left - Helianthus mollis x, M - DNA ladder; 1-9 right - Helianthus tuberosus 6)

Beside the fragments 500 bp, 620 bp, 1000 bp, 1100 bp and 1200 bp that were present in all tested plants, fragments 370 bp and 550 bp were missing in sample no. 5, while a fragment 600 bp was missing in sample no. 3. Fragment 420 bp was missing in samples 4 and 9, while fragments 220 bp and 250 bp occurred only in these samples. Fragment 640 bp was amplified only in samples 3 and 4. In the accession *H. mollis* 1298 there was eight uniform fragments: 2000 bp, 1500 bp, 1300 bp, 1200 bp, 900 bp, 800 bp, 670 bp and 650 bp (Figure 6). Fragment 670 bp was uniform in the accession of *H. mollis* 1298, and polymorphic in the accession *H. mollis* x, while the fragments 1200 bp and 650 bp were detected in both *H. mollis* accessions. The same primer in accession *H. rigidus* 1844 amplified uniform products size of 950 bp, 1100 bp, 1400 bp, 1600 bp and 2200 bp (Figure 6). In accession *H. tuberosus* 6, after amplification with primer C04, in all samples were found seven fragments (220 bp, 600 bp, 650 bp, 950 bp, 1100 bp, 1500 bp and 1700 bp), while fragments 500 bp, 550 bp and 700 bp were polymorphic (Figure 4).

On RAPD profile of accession *H. mollis* 1298, obtained with primer C15 all fragments (2000 bp, 1900 bp, 1800 bp, 1100 bp, 900 bp, 700 bp, 500 bp and 300

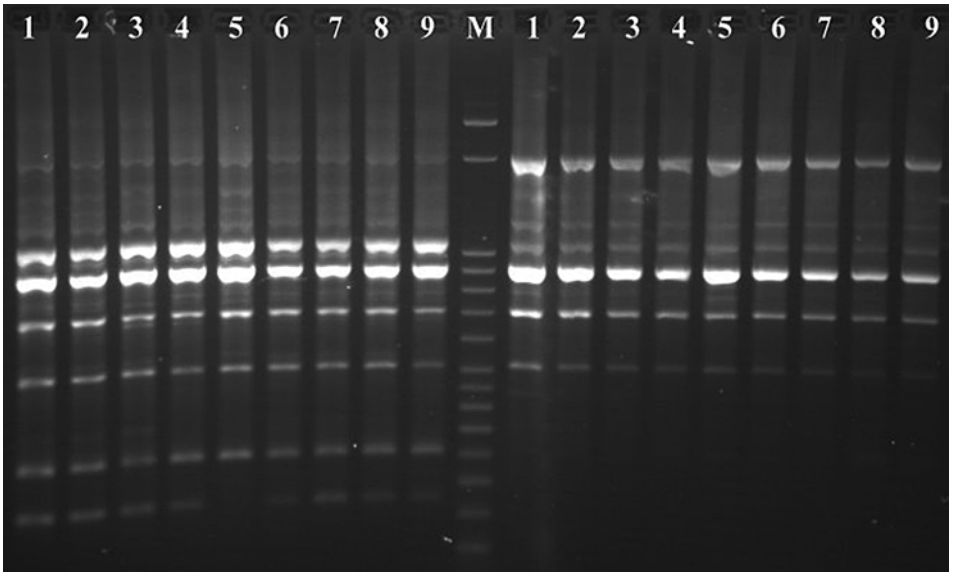

Figure 5. RAPD profile obtained with C15 primer
(1-9 left - Helianthus mollis x, M - DNA ladder, 1-9 right - Helianthus tuberosus 6)

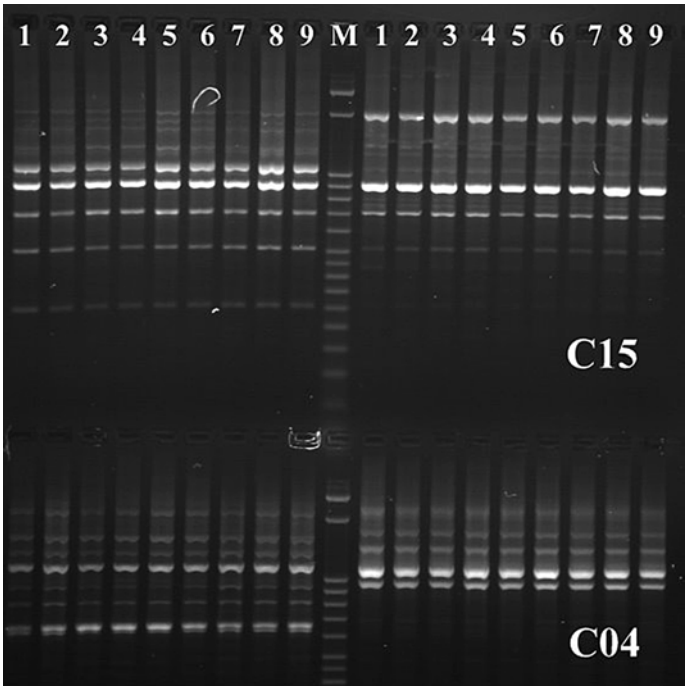

Figure 6. RAPD profile obtained with C15 and C04 primers
(1-9 left - Helianthus mollis 1298, M - DNA ladder, 1-9 right - Helianthus rigidus 1844)

bp) were uniform (Figure 6). The fragments of the same size as in *H. mollis* 1298 (2000 bp, 1100 bp, 900 bp, 700 bp, 500 bp and 300 bp) were observed on RAPD profiles of *H. mollis* x, with the exception of the polymorphic fragment 220 bp and fragments of very low intensity with size 1900 and 1800 bp (Figure 5). In accession *H. tuberosus* 6, fragments 500 bp, 700 bp, 900 bp, 1100 bp, 1200 bp and 2000 bp could be detected in all samples (Figure 5). All samples of accession *H. rigidus* 1844 had fragments 300 bp, 420 bp, 500 bp, 700 bp, 900 bp, 1100 bp and 2000 bp (Figure 6).

Comparing the common fragments obtained in reactions with primer C15 in both accessions *H. mollis* with RAPD profiles of accessions *H. tuberosus* 6 and *H. rigidus* 1844, it was noticed that the 2000 bp fragment was present in all analyzed accessions, but, in *H. tuberosus* 6 and *H. rigidus* 1844 the amount of obtained PCR product was much higher than in *H. mollis*. The intensity of 1100 bp fragment, present in all examined accessions, was much lower in samples of susceptible accessions *H. tuberosus* 6 and *H. rigidus* 1844 than in both *H. mollis* accessions.

The analysis of RAPD profiles obtained in reactions with primer C04 showed that DNA fragments 1200 bp and 670 bp were present only in accessions of *H. mollis*. It was also noticed that the fragment 650 bp, detected in *H. mollis* accessions, was not detected in *H. rigidus* 1844 and in the samples of *H. tuberosus* 6 the intensity of obtained band was very low.

Table 2: Association of loci with level of stem rot tolerance in all tested accessions expressed by contingency coefficients

Loci[1]	Tolerance (%)		Loci[1]	Tolerance (%)	
	100	0		100	0
C04-220bp	-	0.390	C04-1200bp	0.707	-
C04-370bp	0.471	-	C04-1300bp	0.5	-
C04-420bp	0.441	-	C04-1400bp	-	0.5
C04-550bp	0.349	-	C04-1600bp	-	0.5
C04-620bp	0.5	-	C04-1700bp	-	0.5
C04-650bp	0.5	-	C04-2000bp	0.5	-
C04-670bp	0.553	-	C04-2200bp	-	0.5
C04-700bp	-	0.471	C15-220bp	0.471	-
C04-800bp	0.5	-	C15-300bp	0.5	-
C04-900bp	0.5	-	C15-420bp	-	0.5
C04-950bp	-	0.707	C15-1200bp		0.5
C04-1000bp	0.5	-	C15-1800bp	0.5	-
C04-1100bp	-	0.5	C15-1900bp	0.5	-

[1]Only loci that showed significant association with tolerance or susceptibility are presented in tabl

In order to check and statistically confirm if there was significant association of specific fragments either with resistance or susceptibility, contingency coefficient test was done (Table 2). This test was used by Hassan *et al.* (2011) for identifying genetic markers for *Orobanche* resistance in sunflower, as well as Muller *et al.*

(2009) for association of traits among and within sunflower populations. Out of 36 fragments generated, 26 were significantly associated with resistance/susceptibility. As in work of El Sayed Abdel Raouf Sadek Hassan *et al.* (2011), contingency coefficients enabled us to single out two specific fragments that had the higher contingency coefficient values, significantly associated with either susceptibility or resistance to mid-stalk white rot. Fragment C04-950 bp was found to be significantly associated to susceptibility, as it was generated only in susceptible accessions, while fragment C04-1200 bp was found to be associated with the resistance, as it only occurred in *H. mollis* accessions.

According to the presented results, the best candidates for potential markers for resistance to *Sclerotinia* are fragments 1200 bp and 670 bp, obtained in reaction with primer C04. These fragments were present in both resistant accessions of *H. mollis*, but in the accessions of *H. rigidus* 1844 and *H. tuberosus* 6 were not observed. The fragments that could also be interesting as potential markers are 2000 bp and 1100 bp, obtained in reactions with C15 primer, as well as fragment of 650 bp, from reactions with C04. Although these fragments were present in both resistant and susceptible accessions, the difference in the intensity of the bands was significant. The selected markers should be checked and results verified in further studies including higher number of wild sunflower accessions differing in their reaction to stem white rot caused by *Sclerotinia.*

ACKNOWLEDGEMENT

This work was supported by Ministry of Education and Science, Republic of Serbia, project TR 31025.

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