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Use of Molecular Marker for Assay Gene Dosage Resistant Gene to Rhizomania Disease (Rz_1) in Sugar beet (*Beta vulgaris* L.)

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Abstract: This study verified the effect of infected soil on resistant plants varieties to rhizomania disease and with using a molecular marker discriminated homozygote from heterozygote genotypes which this theory performed with use of infected soil that was provided from infected fields then greenhouse test was done to identify resistant and susceptible plants. Extracted DNA from leaves of resistant and susceptible plants was bulked to provide two bulks for resistant and susceptible plants then verified affect of allele (R) on increase resistance to Rhizomania disease. The F_2 population for study obtained from cross between Holly1-4 as resistant parent and annual cultivar as susceptible parent. In this study three-hundred RAPD primers was used for detection of molecular markers linked to resistance gene. Finally the gene (Rz_1) was tagged with using of two RAPD primers and one of the markers was OP-AN_9 which was mapped 13.7 cM apart from Rz_1 gene in repulsion phase. Then with t-test statistical method and repulsion marker verified equal of variations related to two population heterozygote and dominant homozygote genotypes. Finally accepted hypothesis equal of variation between two population at $p \leq 0.05$. This result show don't effecting additional one allele (R) to resistance again Rhizomania disease.

Key words: Sugar beet, Rhizomania disease, resistant gene, gene dosage

INTRODUCTION

The Rhizomania disease is the important disease sugar beet in world and is reported from USA, European countries and many countries of Asian including Iran (Lennefors *et al*., 2000; Wisler *et al*., 1997; Nielsen and Nicolaisen , 2001). This disease affect on decrease yield sugar in field (Wisler *et al*., 1997; Johnsen *et al*., 1995). Rhizomania refers to the excessive hairy root proliferation that results from infection by the *Necrotic yellow vein virus* (BNYVV, genus Benyvirus). This virus transmitted by the soil-borne plasmodiophoride-like fungus, *Polymyxa betae* Keskin (Tamada *et al*., 1990; Keskin, 1964). Source of resistance to rhizomania were found in Holly sugar beet company source (Lewellen *et al*., 1987). Resistance in Holly is simply inherited by a single dominant gene (Rz_1) (Lewellen *et al*., 1987; Scholten *et al*., 1996, 1997; Barzen *et al*., 1997; Readfearn and Asher, 1997; Asher and Kerr, 1996). Also resistance to BNYVV has been obtained in several Wild Beet (WB) accession *B. vulgaris* subsp. *maritima* originally collected from France, UK, Denmark and Italy (Whitney, 1989; Lewellen, 1995). For example WB42 is thought to have been originally collected in Denmark by Viggo Lund in the 1950 (Lewellen, 1995). Resistance in WB42 is inheritance by another dominant gene (Rz_2) (Lewellen, 1995; Scholten *et al*., 1997; Francis *et al*., 1998; Francis, 1999). In this study, we verified the effect of infected soil on resistant plants varieties to rhizomania which this theory performed with use of infected soil that was provided from infected fields.

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MATERIALS AND METHODS

Studies on the inheritance of resistance to BNYVV were performed in greenhouse of Sugar Beet Seed Institute (SBSI) in Karaj, Iran (2004) with the resistant sugar beet accession *B. vulgaris* subsp. *vulgaris* Holly1-4, which is a selection from the Holly source (Lewellen *et al*., 1987) and the resistant wild beet accession *B. vulgaris* subsp. *maritima* WB42. Both accessions are diploid with 2n = 18. Plants of the resistant wild beet accession WB42 also were crossed in pairs with susceptible sugar beet germplasm 261 and annual beet accession. Resistant F_1 plants obtained after crosses of Holly1-4 with the susceptible sugar beet germplasm 261 and annual beet accession were selfed to product F_2 seeds.

Greenhouse Test and ELISA Test

A greenhouse test for screening sugar beet for resistance gene to BNYVV (Paul *et al*., 1992) was used in the present study. Seed sowed in autoclaved (121°C, 1.5 h) sand. Seedlings were transplanted at 4 leaf stage to uniform mixture of infested soil was collected from Shiraz, Iran that contained the A type of BNYVV. Regina germplasm was used in all of the tests as negative and positive control. To produce negative control, seedlings of *Beta vulgaris* sugar beet cultivar Regina were transplanted into sand (not artificially infected plants as negative controls).

To produce positive controls, seedlings of Regina were transplanted into an uniform mixture of infested soil and sand (3:7 V/V). Infected plants as positive controls. Plants were watered twice a week with 30 mL of 0.2 diluted (Hoagland and Arnon, 1950) solution (pH 7.0). Inoculation experiments were performed in the greenhouse at 25/17°C (day/night). Rootlets were analysed for the virus by standard Double Antibody Sandwich ELISA (DAS-ELISA) as was described by Clark and Adams (1977). This experiment was used a commercial polyclonal antiserum and BNYVV infected *N. clevelandii* leaf (Bioreba AG, Switzerland). The 60 internal wells of micro titer plate (polystyrene Nunce. Flat bottom 442404) were used for ELISA, while the outer wells were filled with PBS-Tween 20 during all steps of the ELISA procedure. All samples were read by Lab system Multiskan EX 355 at 405 nm. Plants were considered susceptible if their samples would show an absorption value more than two time value of the negative control.

DNA Extraction

Genomic DNA was extracted from frozen leaves from individual plants of F_2-A1-110 and F_2-93 populations following the procedure of Saghai-Maroof *et al*. (1984). DNA concentrations were estimated by spectrophotometery, after that PCR was performed in a total volume of 25 μL containing, 50 ng genomic DNA, 0.2 mM each of dATP, dCTP, dGTP, TTP, 25 ng primer (Operon kit), 2.5 μL 10x Reaction buffer (100 mMTris-HCl, pH = 9; 500 mM KCl), 1 unit Taq Polymerase (Smar Taq). DNA amplification was performed in a thermocycler (Biometra T3) in PCR reaction tubs. The thermal cycles used were: 1 cycle of 5 min at 94°C, followed by 40 cycle of 45 sec at 94°C, 45 sec at specific temperature and 80 sec at 72°C, then finally 1 cycle of 10 min at 72°C for final extension. Amplified products were separated by gel electrophoresis using 1.2% agarose gel with TAE buffer and staining with ethidium bromide.

Bulk Segregant Analysis (BSA)

BSA technique (Michelmore *et al*., 1991) was performed on bulks of DNA of 10 of the most resistant (with virus concentration maximum 0.3 ng mL^{-1} virus) and of the most susceptible plants (with virus concentration at least 0.6 ng mL^{-1} virus) for any population. Primers which had amplified

a DNA fragment in only one of the bulks were confirmed on the same set of bulk, followed by PCR on ten individual resistant and ten susceptible plants. RAPD markers with the best linkage to resistance gene were evaluated further on additional number of individual plants.

RESULTS AND DISCUSSION

To identify RAPD marker linked to genes for resistance to BNYVV (Merdinoglu *et al*., 1995), DNA bulks were found to be mixture of the most resistant and most susceptible plants of the segregating families of Holly1-4 and WB42 for each set of collection 300 Operon primers were screened. Between 10-20 primers amplified RAPD markers both in a resistant and a susceptible bulk. These primers were examined further on individual plants. The primer Op-AN_9 had generated DNA fragment that was found to be linked to the susceptible locus in F_2-A1-110 population and primer Op-X_9 that had generated DNA fragment 1150 base pair, but no primers found for F_2-93. For this reason, we stopped investigation on F_2-93 population and continued this study on F_2-A1-110 population and primer Op-AN9 was examined on individual plants.

The marker Op-AN9 amplified 600 bp fragment that was linked to Rz_1 gene. This marker was mapped for Rz_1 gene in 13.7 cM (centi Morgan) apart from Rz_1 gene in repulsion phase by Mapmaker Software version 3.0. In study of Barzen *et al*. (1997) and Scholten *et al*. (1996) this distance was less of this results because method which they used for estimation of distance was relative of plants that didn't have 600 bp band on gel electrophoresis to total plants while in our study distance was estimated as relative of two-time of plants that didn't have 600 bp band on gel electrophoresis to total of plants. In fact we considered heterozygote genotypes in our estimation for more precision. Also plants that were used by Scholten *et al*. (1996) were 60 individual plants while in our study they were 160 individual plants. This results was supported by Amiri *et al*. (2003). Also soil that we used concentration of virus was more than soil that was used by Scholten *et al*. (1996) and Amiri *et al*. (2003), thus we should have increased threshold of resistance between susceptible and resistance plants that this threshold is less in study of Scholten *et al*. (1996) and Amiri *et al*. (2003).

Study Effect of Gene Dosage Related to (R) Allele

With using of information related to this marker and results of ELISA test, it was possible to discriminate between dominant homozygous and heterozygous genotypes. Accordingly, this marker was evaluated on 30 individual of resistant plants of F_2-A1-110 population and discriminated dominant homozygous and heterozygous plants. Then different absorbance means of ELISA test related to both dominant homozygous and heterozygous plants were evaluated with T-test statistical method by computer programming MSTATC (Table 1). First hypothesis was equality absorbance means both dominant homozygous and heterozygous plants. This hypothesis at $p \leq 0.0\ 5$ was accepted. As regard to results, we concluded that the presence of a resistant allele has not effected on resistance increase to disease and both Rz_1Rz_1 and Rz_1rz_1 genotypes were equal in resistance. Also this result demonstrated the dominant character of the resistance. Present results were supported by Scholten *et al*. (1996) and Amiri *et al*. (2003).

Table 1: Mean of absorbance (ELISA) for Rz_1Rz_1 and Rz_1rz_1 genotypes with use from Op-$AN9_{600r}$ marker in F_2-A1-110 population

Marker	Mean of absorbance Rz_1 Rz_1 genotype	n_1	Mean of Rz_1 rz_1 genotype	n_2	T -test
Op-AN9	0.11505	21	0.12189	9	-1.794^{NS}

n_1: No. of homozygous genotype, n_2: No. of heterozygous genotype, NS: Non-Significant

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