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Screening of Sugar Beet Tissue Culture Clones for Resistance to Rhizomania Disease

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Abstract: In this study, sugar beet tissue culture clones were used to screen rhizomania resistant genotypes. At first, explants derived from shoot tips of sugar beet seedlings were transferred to shoot tip elongation media after surface sterilization. Then, the grown shoots were transferred to media containing various hormonal combinations NAA, BA, IBA and GA_3 for multiplication, growth and rooting. Later, the clones were transferred to soil-peatmoss mixture were adapted to greenhouse conditions. For screening clones against rhizomania, the genotypes of adapted clones were selected and inoculated to rhizomania-infested soil. This experiment was in a randomized complete block design with three replicates (three inoculation times) in greenhouse. Adapted plants were transferred to the soil containing rhizomania virus. All infested soils were diluted 3 to 7 with sand. After two months, infested plants were examined by DAS-ELISA test also optical densities of the samples were analyzed by SAS program. Significant differences among genotypes and blocks were observed. Genotypes were classified to few groups (ranked from completely susceptible to completely resistant). The difference between blocks was because of difference of inoculation time temperature. Use of clones of each genotype caused an increase in selection accuracy of resistant genotypes. By use of this method, chance of escaping from inoculation factor decrease and researchers can determine to be resistance of plants with high level of confidence and apply in breeding programs.

Key words: Sugar beet, rhizomania, tissue culture clone, ELISA

INTRODUCTION

Rhizomania is the most important and damaging sugar beet disease which nowadays, decreases yield 30-100% (Lennefors *et al*., 2005). It exists in most fields of Europe, Asia and America (Koeing and Lennefors, 2000). In Iran, this disease firstly was reported in 1995 in sugar-beet growing areas in Fars province and then it was affirmed in most sugar-beet fields in Iran (Izadpanah *et al*., 1996). It is a severe disease of sugar beet caused by *Beet necrotic yellow vein virus* (BNYVV), which is transmitted by soil inhabiting pathogen *Polymyxa betae Keskin* (Richards and Tamada, 1992). Since the agronomical, biological and chemical disease control is not economical and effective, therefore breeding for rhizomania resistance cultivars by classical and modern plant breeding programs to control the disease and continuing of sugar beet products is so important for sugar beet seed institutes and companies (Biancardi *et al*., 2002). Sugarbeet, *Beta vulgaris* L., is a naturally cross-pollinating biennial plant and modern genotypes are highly heterozygous. Therefore, the generation of new varieties by conventional breeding practices is slow (Lindsey and Gallois, 1990). Alternative method for sugarbeet improvement takes advantage of biotechnology to propagate genetically identical plants via tissue culture, speed up classical breeding programs with the use of molecular markers, or transfer desired genes into a transformable sugarbeet line.

The only way to maintain profitable sugar beet production in fields infected by BNYVV is to use resistant sugar beet cultivars. The resistance present in most of today's resistant varieties is based on the Holly source (Biancardi *et al*., 2002). To produce disease resistant varieties such as rhizomania in sugar beet, at first we need to screen resistant genotypes then use them in crossing process. Lein *et al*. (2006) determined location of rhizomania resistance loci by use of sugar beet tissue

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culture clones. The origin of these clones was F2 single plant population which resulted from crossing between rhizomania resistant and susceptible sugar beet plants. Since in greenhouse evaluation for rhizomania resistance, some susceptible genotypes during infection escape from inoculums and show apparently resistance and make problems in the breeding process. So in this study, sugar beet tissue culture-clones with same genotypes were used in greenhouse evaluation of resistance to rhizomania and resistant plants screened based on the mean of ELISA absorption of clones in each genotype. Therefore, the aim of this study is to obtain one reliable method for screening of single plants resistance to rhizomania.

MATERIALS AND METHODS

Plant materials: The plant material was the sugar beet tetraploid seed bulk of rhizomania resistant parents. This study has been done from March to December 2006 in sugar beet seed Institute, Karaj, Iran.

Preparing of tissue cultured-clones: seeds were surface sterilized by common methods in the lab and then cultured in water-agar media. After 2 weeks, the shoot tips excised from seedling and transferred to MB medium containing MS (Murashige and Skoog, 1962) salts and B5 (Gamborg *et al*., 1968) vitamins along with BA and GA_3 hormones with concentrations of 0.5 and 0.01 mg L^{-1} respectively. Propagation of derived shoots was done in MB medium containing hormonal composition of BA, NAA and IBA with concentrations 0.5, 0.1 and 0.1 mg L^{-1} respectively. MB medium was used to grow shoots and MB medium containing of 1.5 and 1.5 mg L^{-1} NAA and IBA respectively for rooting the shoots. Growth chamber conditions were 16 h light and 8 h dark with 50-60 μmol m^{-2} sec^{-1} and 22-24°C temperature. After rooting, the tissue cultured-clones were transferred to pots containing sterilized soil and peatmoss with ratio of 1:1 and irrigated with Hoagland solution (Hoagland and Arnon, 1950) for adaptation to greenhouse conditions (Fig. 1).

Inoculation of clones with rhizomania infested-soil: Two-month old clones were used for evaluation of resistance to rhizomania in greenhouse. For this, 11 genotypes were selected and used in randomized complete block design (RCBD) with 3 replicates (3 inoculation times) and 3 samples in each replicate. Totally, 9 clones from each

Fig. 1: Tissue culture clones of sugar beet (a, b) rooting and (c) adapted to greenhouse condition

genotype were applied. For this, the clones were transferred into the new pots that contained infested soil mixed with sterile soil (3:7) and kept for two months in greenhouse conditions.

ELISA test: BNYVV was tested by enzyme-linked immunosorbent assay (ELISA). The clones root was harvested 2 months after inoculation period. 0.1 g from each sample root was weighed and sap was extracted using potato peeler and diluted in 1.5 mL ELISA extraction buffer then ELISA process was done with DAS-ELISA (double antibody sandwich ELISA) method (Clark and Adams, 1977). A healthy control plant sap was included in seven wells in each plate as a negative control and one well included infested plant sap as positive control (BNYVV infested Nicotiana leaf extraction which was supplied from Bioreba AG. Switzerland). Then ELISA optical densities (OD) were recorded using Multiskan RC microplate (Thermo Labsystems, Helsinki, Finland) ELISA reader at a wavelength of 405 nm (A_{405}) 20 min after addition of substrate (*p*-nitrophenyl phosphate) and incubated at room temperature.

Statistical analysis: ELISA reading absorbance was transformed for a more normal distribution and then was analyzed in SAS program. Duncan's multiple range test was used to compare the means of virus titers.

RESULTS AND DISCUSSION

ELISA values (Table 1) showed significant difference between genotypes (p = 0.01) and between blocks (p = 0.05). Duncan method classified blocks in 0.05 probability level to different groups which justified based on 3 inoculation times and temperature condition in each block result of which was shown in Table 2. Despite, genotypes screening process had high level of accuracy because from each genotype we prepared 9 same genetically clones and inoculated with rhizomania infested-soil and their resistance levels were evaluated quantitatively.

According to Table 3, first to eleventh treatments were average of screened genotypes for resistance, 12th treatment was average of negative control that cultured in sterile soil. 13th treatment was infection OD value or positive control. 14th and 15th treatments showed infection and sensitive extent in regular.

According to ELISA response intensity difference in various experiments makes the necessity for a scale which can distinguish healthy samples from infested sample. Hill and Jackson (1984) presented a formula to calculate infection range as following $R = \bar{X} + 3SD$ in this formula R was infection range, $\bar{X}$ was mean of OD of negative control, SD: was standard deviation of OD value of negative control. Based on Table 3 range of ELISA absorption (OD) for genotypes was 0.131-1.409 and based

Table 1: The sources of variation for ELISA data

Source of variation	Degree of freedom	MS§
Block	2	1.5397*
Genotype	10	1.5177**
Error	20	0.4316
Total	32	

*Significant at 5% probability ($p \leq 0.05$) **Significant at 1% probability ($p \leq 0.01$) §Mean squares

Table 2: Differences among ELISA OD Blocks*

Block	Inoculation time	No. of sample	Mean of OD	Mean of greenhouse temp.	Duncan grouping*
I	13 May-14 July	33	0.7788	35°C	a
II	19 June-20 August	24	0.7775	37°C	a
III	30 July-30 September	26	0.4540	29°C	b

(*$p \leq 0.05$)

Table 3: Duncan multiple test for rhizomania screening genotypes ($p \leq 0.05$)

Genotype name	Genotype No.	No. of sample	Mean of ELISA OD	Duncan grouping	Effect response
RB-24	6	8	1.4089	a	Completely susceptible
RB-11	2	8	1.2651	a	Completely susceptible
RB-4	1	9	1.081	a	Completely susceptible
RB-30	7	9	0.8925	ab	Susceptible
RB-15	4	7	0.7142	abc	Semi susceptible
RB-33	8	9	0.6602	abc	Semi susceptible
RB-41	9	7	0.5777	abcd	Partially susceptible
RB-81	11	7	0.5212	abcd	Partially susceptible
RB-20	5	6	0.3601	bcd	Semi tolerance
RB-13	3	7	0.2182	cd	Tolerance
RB-45	10	6	0.1311	d	Resistant
Healthy control	12	9	0.1284		
Positive control	13	2	2.285		
Threshold of infection	14	$\bar{X} + SD$	0.145		
Threshold of susceptible	15	$2\bar{X}$	0.232		

(*$p \leq 0.05$)

on above formula infection scale calculated 0.145 which means the genotype that had lower OD than 0.145 was healthy and resistant.

Grimer *et al.* (2007) was calculated distribution of ELISA OD in sugar beet single plants for BNYVV in F2 bulk and mean of ELISA OD of 10 uninfected control plants was 0.12 and ELISA value of the infected individuals range from 0.147-2.76 that is similar to this study results.

Lein *et al.* (2006) determined location of rhizomania resistance loci by use of sugar beet tissue culture clones. The origin of these clones was F2 single plant population which resulted from crossing between rhizomania resistant and susceptible sugar beet plants. For this, they used mean of 7 ELISA OD of same genetically plants instead of single plant ELISA OD. The range of genotype ELISA OD was 0.8-3.4 which was read 2 h after incubation by ELISA reader. But in this study ELISA reading was done 20 min after incubation, according to relationship between absorption amounts during incubation, therefore in this experiment decrease in amount of ELISA OD range was justified. Also Amiri *et al.* (2003) determined threshold of susceptible plants from resistant plants by $2\bar{X}$. Based on Table 3, genotypes located in range of completely susceptible to resistant. In this study since genotypes were tetraploid and contained different number of resistance controlling alleles (gene dosage effect), (RzRzRzRz>RzRzRzrz>RzRzrzrz>Rzrzrzrz>rzrzrzrz). We found extreme rang of ELISA OD value in various genotypes. This result confirmed the Amiri *et al.* (2003) results.

Wisler *et al.*(1999) demonstrated that triploid varieties carrying *Rz1* become more heavily infected with BNYVV than diploid varieties. They declared that dose (number of allele in variety) and ratio of Rz to rz in variety can influence in general performance of sugar beet cultivars in rhizomania infection condition. They compared diploid, tetraploid and triploid sugar beet in range of ELISA OD in 405 nm by TAS-ELISA method. The result showed that absorption value (OD) is related to dose and ratio of effective alleles. Tetraploid hybrids Rzrzrzrz had higher absorption value than Rzrz absorption amount.

The most important factors of infection are soil temperature and humidity (Tuitert and Hofmeester, 1992). Therefore based on Table 2 according to different temperatures between first replicate (block) with average of 37°C (May to July), that had maximum average temperature and second replicate with average of 35°C (June to August) and third replicate with average of 29°C (August to October) evaluated by Duncan's Multiple Range Test ($p = 0.05$). First and second replicate located in a group and third replicate determined as b group. Difference between replicates 1 and 2 with replicate 3 resulted from temperature difference in greenhouse conditions, present results for the Duncan grouping was similar to Tuitert and Hofmeester (1992) results.

There can be many explanations to why plants susceptible to rhizomania can be found in the resistant sugar beet varieties. In this research by using tissue cultured clones and several replicate and also high level infection soils, chance of escape from pathogen is decreased.

Therefore screening of tissue culture clones in disease resistance evaluation compared with usual screening method had high level of accuracy.

By use of this method, chance of escaping from inoculation factor decrease and researchers can determine to be resistance of plants with high level of confidence and apply in breeding programs. Therefore in breeding programs that involve tissue culture clones it seems to identify resistant plants is done according to mean of ELISA some clones from each genotypes than only single plant evaluation.

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REFERENCES

Amiri, R., M. Moghaddam, M. Mesbah and S.Y. Sadeghian, 2003. The inheritance of resistance to *Beet necrotic yellow vein virus* (BNYVV) in *B. vulgaris* subsp. Maritime, accession WB42 statistical comparison with Holly-1-4. Euphytica, 132: 363-373.

Biancardi, E., R.T. Lewellen, M. De Biaggi, A.W. Erichsen and P. Stevanato, 2002. The origin of rhizomania resistance in sugar beet. Euphytica, 127: 383-397.

Clark, M.F. and A.N. Adams, 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol., 34: 475-483.

Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirement of suspension cultures of soybean root cells. Exp. Cell Res., 50: 151-158.

Grimmer, M.K., S. Trybush, S. Hanley, S.A. Francis, A. Karp and M.J.C. Asher, 2007. An anchored linkage map for sugar beet based on AFLP, SNP and RAPD markers and QTL mapping of a new source of resistance to Beet necrotic yellow vein virus. Plant Breed., 114: 1151-1160.

Hill, S.A. and E.A. Jackson, 1984. An investigation of the reliability of ELISA as a practical test for detecting *Potato leaf roll virus* and *Potato virus* Y in tubers. Plant Pathol., 33: 21-26.

Hoagland, D.R. and D.I. Arnon, 1950. The water culture method for growing plants without soil. Cal. Agric. Exp. Sta. Circ., 32: 347-347.

Izadpanah, K., P. Hashemi, R. Kamran, M. Pakniat, A. Sahandpour and M. Masoumi, 1996. Widespread occurrence of rhizomania-like disease of sugar beet in Fars. Iran. J. Plant Pathol., 32: 155-157 (In Persian, English Summary).

Koeing, R. and B.L. Lennefors, 2000. Molecular analyses of European A, B and P type sources of *Beet necrotic yellow vein virus* and detection of the rare P type in Kazakhstan. Arch. Virol., 145: 1561-1570.

Lein, J.C., K. Asbach, Y. Tian, D. Schulte, C. Li, G. Koch, C. Jung and D. Cai, 2006. Resistance gene analogues are clustered on chromosome 3 of sugar beet and crosegregate with QTL for rhizomania resistance. Genome, 50: 61-71.

Lennefors, B.L., E.I. Savenkov, S.B. Mukasa and J.P.T. Valkonen, 2005. Sequence divergence of four soilborne sugar Beet-infecting viruses. Virus Genes, 31: 57-64.

Lindsey, K. and P. Gallois, 1990. Transformation of sugarbeet (*Beta vulgaris*) by *Agrobacterium tumefaciens*. J. Exp. Bot., 41: 529-536.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol., 15: 473-497.

Richards, K.E. and T. Tamada, 1992. Mapping functions on the multipartite genome of *Beet necrotic yellow vein virus*. Annu. Rev. Phytopathol., 30: 291-313.

Tuitert, G. and Y. Hofmeester, 1992. Epidemiology of *Beet necrotic yellow vein virus* in sugar beet at different initial inoculum levels in the presence or absence of irrigation: Dynamics of inoculum. Neth. J. Plant. Pathol., 98: 343-360.

Wisler, G.C., R.T. Lewellen, J.L. Sears, H.Y. Liu and J.E. Duffus, 1999. Specificity of TAS-ELISA for *Beet necrotic yellow vein virus* and its application for determining rhizomania resistance in field-grown sugar beets. Plant Dis., 83: 864-870.