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Development of tobacco (*Nicotiana tabacum* L.) doubled haploid lines resistant to *potato virus Y* ^{0}

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Abstract

Viral diseases such as *Potato Virus Y* (*PVY*) in tobacco plants can cause a lot of damage annually. In order to obtain *PVY* resistant doubled haploids (DH) of *Nicotiana tabacum* with desirable traits. Two varieties of tobacco VAM resistant to *PVY* and K326 of recognized high quality of leaves were crossed. The F_1 hybrids were grown in pots and the anthers were cultured on PGR-free MS medium. After production of somatic embryos, colchicine treatment (0.4%) was applied for 8 hours. Thereafter, 184 plantlets were transferred to greenhouse and then moved to the field and self-pollinated. The derivatives of colchicine treated and fertile plants were inoculated with *PVY*^{0}. DAS-ELISA technique and rating of symptoms were used to select resistant plants. From a total of seventeen DH, seven plants were selected as pure lines and evaluated under field condition. The DH lines showed significant differences between them for morphological and chemical traits. Some of the DHs had longer and wider leaves than K326 cultivar as control. The content of sugars was not affected by genotype. Finally, three doubled haploid lines (TDH-03, TDH-09, TDH-11) with desirable characteristics were selected as *PVY* resistance.

Key words: Tobacco breeding, doubled haploid, *potato virus Y*, ELISA, GGE biplot

Introduction

Viral diseases that causes severe damage to tobacco (*Nicotiana tabacum* L.) plants usually, decrease the tobacco production worldwide. *Potato virus Y* (PVY) is one of the most important virus in tobacco (Quenouille et al. 2013). It can cause brown-rib and veinal necrosis as well as disturbances in plant metabolic pathways (Verrier et al. 2001). Development and cultivation of varieties with high level of resistance is the most efficient and practical way of protection against PVY. The well known source of resistance against this virus is "Virgin A Mutant" (VAM) cultivar. It was obtained by X-ray irradiation that caused a deletion in a gene for susceptibility to Potyviruses. Doroszewska and Chrzanowska (2001) studied various sources of resistance to different isolates of PVY and announced that the VAM cultivar was not infected by Y^N, Y^0, Y^{Wi}, Y^{Esp} isolates. For this reason, the *va* gene of VAM cultivar has been used for breeding of some cultivars, such as TN86 and NC745 (Burk et al. 1979; Miller 1987). However, resistance in these cultivars was lower compared to VAM (Acosta-Leal and Xiong 2008). Apart form *va* gene that mainly prevents the systemic movement of PVY, there is another gene *Rvam2* that prevents the virus accumulation in the infected tissues (Acosta-Leal and Xiong 2008). Furthermore, a cytoplasmic factor in VAM reduces the frequency of resistance-breaking virus variants. Therefore, the PVY virus resistance can be increased by inserting further factors during the breeding of new cultivars.

Anther culture and creation of doubled haploids (DH) are useful methods to achieve resistant lines in minimum time, through transfer of target gene(s). One of the major advantages of doubled haploid plants is reducing the time in obtaining genetically pure, completely homozygous lines that possess desired traits. Nichols and Rufty (1992) produced tobacco

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doubled haploids which were resistant to black shank. Some of the researchers reported same information on other plants and pathogens (Bekkuzhina and Kalashnikova 2009; Wenzel and Uhrig 1981). Selection for disease resistance in doubled haploid plants is easier as compared to F_2 population obtained by conventional intervarietal breeding (Trojak-Goluch and Berbeæ 2011). Ciuperca et al. (2003) applied tissue culture and androgenic techniques to combine resistance to PVY and TMV in tobacco breeding lines. The authors produced new Virginia and Burley cultivars of tobacco using this approach (Ciuperca et al. 2003).

Induction of doubled haploids can be obtained through long term tissue culture and plant regeneration from spontaneously diploidized tissue (Czubacka et al. 2012), or through different colchicine treatments of generative organs. Using *in vivo* colchicine treatment of caraway flower umbels, Smýkalová et al. (2009) received a numerous population of doubled haploids. Application of 0.2 % colchicine solution to cotyledonary embryos of tobacco resulted in obtaining fertile DH lines (Patrascu et al. 1999). However, the highest percentage of diploid plantlets were produced from culture of tobacco anthers that were treated with 0.4 % colchicine for 8 hours before planting (Takashima et al. 1995).

Fast and reliable methods for ploidy determination are essential for a breeding process. Some of the methods include chromosome counting in somatic cells, pollen grain size and also the stomata size (Dhooghe et al. 2011). Some of doubled haploid related traits have been reported recently, such as number of chloroplast in stomata cells (Solov'eva 1990), the size of stomatal cells (Sood et al. 2003) and the number of stomata of the leaf (Rauf et al. 2006). The authors reported that stomata cells measurements were one of the most efficient and optimized methodologies (Sood et al. 2003; Rauf et al. 2006).

The aim of the present study was to produce doubled haploids with resistance to PVY and other desirable agronomic traits of cultivar K326.

Materials and methods

Plant materials

The plant material in the study include VAM cultivar carrying, *va* gene as well as a target factor in the cytoplasm (Acosta-Leal and Xiong 2008) was used as a female parent and cultivar K326 as a male parent. A cross was made between them manually F_1 seeds. The, F_1 hybrids were planted to maturity and flowers were selected for anther culture.

Anther culture

The flowers of F_1 hybrids were subjected to pre-chilling treatment for 10 days at 7-8°C according to Chawla (2003) method. Flowers were disinfected initially in 70% alcohol for 1 min and then in 1% sodium hypochlorite solution for 15 minutes, and finally, rinsed twice in sterile distilled water. Anthers were cultured in the Petri dishes containing Plant Growth Regulator (PGR)-free MS medium with pH = 5.8 (Chawla 2003) as also follows in the previous study (Shahadati Moghaddam et al. 2006). The Petri dishes were kept in dark conditions at 26±1°C temperature. When the microspore embryos were observed, the Petri dishes were kept in growth chamber under a 16/8 h light/ darkness regime at 26±1°C temperature.

Colchicine treatment

The 2-3 leaf stage plantlets obtained from microspores, were transferred to 0.4 % colchicine (C3915, Sigma) solution for 8 hours according to methods described by Nakamura et al. (1993) and Takashima et al. (1995), subsequently recultured on PGR-free MS medium at pH 5.8. The cultures were kept at 26±1 °C temperature and 45 µmol $m^{-2}s^{-1}$ light intensity until roots were developed. Well rooted plantlets were transferred to plastic pots filled with peat mix and kept in the greenhouse conditions. As soon as plants reached the flowering stage, they were self-fertilized and seeds were obtained from each doubled haploid plant as a new pure line.

Flow cytometry tests

Measurements of DNA content were made using flow cytometry (Partec-PAC) and DAPI (4',6-diamidino-2-phenylindolel, Sigma) staining method (Otto 1990). Leaf samples of 1 cm^2 excised from colchicine treated and non-colchicine treated plants (as internal standard) were mixed and chopped with a razer blade in 0.5 ml of Otto's buffer I. The resulting suspension was filtered and then 2 ml of staining buffer Otto II containing 2 µg/ml DAPI was added. For each sample, about 20000 nuclei were analyzed using a flow cytometer (Partec, Münster, Germany) equipped with a high-pressure 100 W mercury lamp.

Stomatal cell properties

Cells size of stomata of haploid and doubled haploid

plants were studied and compared (Pour Mohammadi et al. 2012). Three plants of both the haploid and doubled haploid were selected and three samples (three microscope slide) were prepared from the leaves of each plant. Three observations were made for each sample. Finally, 27 cells from two groups were compared using t-test.

Determination of doubled haploids resistance

Six plants from each doubled haploid line were cultivated as replications and were inoculated with PVY^0 isolate (as a dominant strain in northern Iran) by leaf rubbing on carborundum dusted plants) by leaf rubbing on carborundum dusted plants. The resistant plants were determined using DAS-ELISA technique (Clark and Adams 1977) by PVY polyclonal antibody (IgG110575, Bioreba) and ranked by outward signs following 0-11 scale given by Verrier et al. (2001) after three weeks. Then, they were compared using completely randomized design.

Field studies

The selected doubled haploid for the assessment of morphological and chemical traits were grown in the field. The entries were grown in complete randomized block design with three replications at 5×8 m^2 plot. Agronomic traits such as length and width of leaf cutters, leaf number, plant height, stem diameter, the fresh and dry yield were measured, including the chemical traits such as nicotine percentage (CORESTA method) (Gorrod and Jacob 1999) and sugar percentage (Bertrand's method) (Chidan Kumar et al. 2012) in 5 plants from each plot.

Statistical analysis

The analysis of variation and means comparison were performed using SAS software. Mean comparison was performed by Duncan test (SAS Inst., 1987). GGEbiplot software (Yan and Kang, 2002) was used to compare growth and quality characteristics between the DH breeding lines and K326 cultivar.

Results

Anther culture

Two hundred anthers were put on MS medium. Microspore embryos were produced in 72 per cent of the total anthers and matured embryos varied from 2 to 13 per anther (Figs. 1a, 1b). Finally, 184 plantlets were obtained. These plantlets were used for producing DH plants by colchicine treatment.

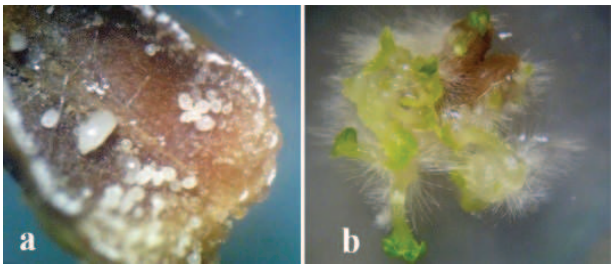

Fig. 1. Microspore embryos of anther culture (a) and haploid plantlets derived from microspores (b)

Colchicine treatment and doubled haploid seeds

Colchicine treatment was performed on 184 plantlets. Plants that survived were transplanted into pots and acclimatized to the greenhouse conditions. Out of these plants, 168 (91%) flowered but large numbers of them did not produced active pollen and out of 24 plants (13 %) produced seed as doubled haploid. Seven seeds were not viable and finally, 17 tobacco doubled haploids (TDHs) lines were obtained (these plants were tested by flow cytometry).

Stomatal cells and flow cytometry tests

T-test (df=27+27-2=52) for length (T=3.32) and width (T=2.89) of stomata cells showed significant differences between haploid and doubled haploid plants ($T_{0.05}$=2.01). But there was no significant difference in length/width ratio of stomatal cells between haploid and doubled haploid plants (T=0.91). The results showed that there were no changes in the general shape of the stomatal cells (Figs. 2a, 2b). Results of

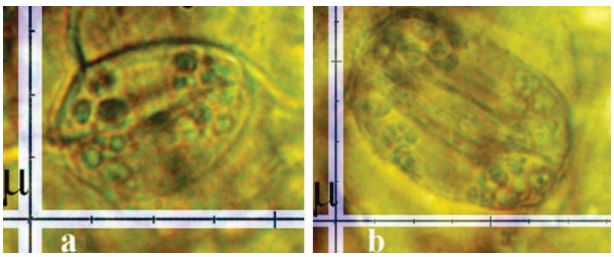

Fig. 2. Stomatal cells of haploid (a) and doubled haploid (b) plants derived from *N. tabacum* cv. VAM x K 326

flow cytometry tests showed that the doubled haploid plants contained twice the amount of DNA compared to haploids (Fig. 4a). Characteristic peaks of the haploid and doubled haploid were placed in channels 31.3 and 62.3 with the coefficient of variation 8.2 and 5.6 percent, respectively.

Determination of resistance in doubled haploids

The assessment of 17 doubled haploid lines in greenhouse conditions revealed a significant difference in DH lines reaction to PVY^0 (Table 1). TDH-06, TDH-09, TDH-12 lines and VAM cultivar had no disease symptoms, whereas TDH-03, TDH-11 and TDH-13 exhibited low level of disease symptoms (Fig. 5). However, K326 cultivar showed severe symptoms of disease and leaf necrosis after three weeks of growing (Figs. 3a, 3b). The resistance of TDH-12, TDH- 09

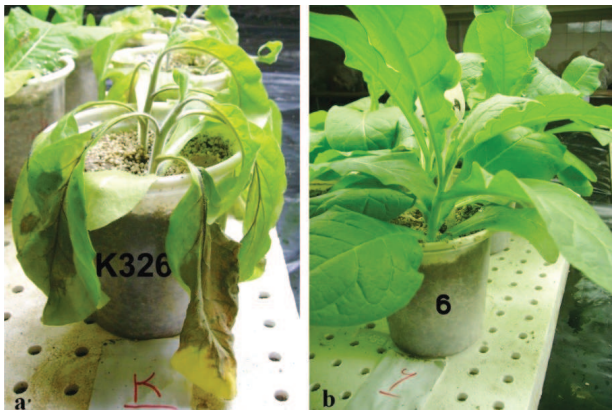

Fig. 3. PVY effects after three weeks of infection. Necrotic symptoms on sensitive *N. tabacum* cultivar K326 (a) (positive control) and a doubled haploid breeding line TDH-06 resistant to PVY (b)

Table 1. Analysis of variance for PVY^0 evidence in the greenhouse condition

Source of variation	df	Mean of squares
Treatment	18	133.18**
Error	90	0.3
Coefficient of Variation (%)	8.8	

**Significant at 1% level of propability

and TDH-06 lines and VAM (control) was confirmed by DAS-ELISA technique using PVY polyclonal antibodies (Fig. 4b). Absorbance values of A_{405} in DAS-ELISA test by PVY polyclonal antibodies confirmed the resistance of TDH-06 (0.024), TDH- 09 (0.029) and TDH-12 (0.032) lines and VAM (0.021) (Fig. 4c). Finally, seven resistant and tolerant lines (TDHs-03, 05, 06, 09, 11, 12 and 13) were selected for field evaluation.

Field studies and line selection

Analysis of variance between seven DH lines and control cultivar showed significant differences between

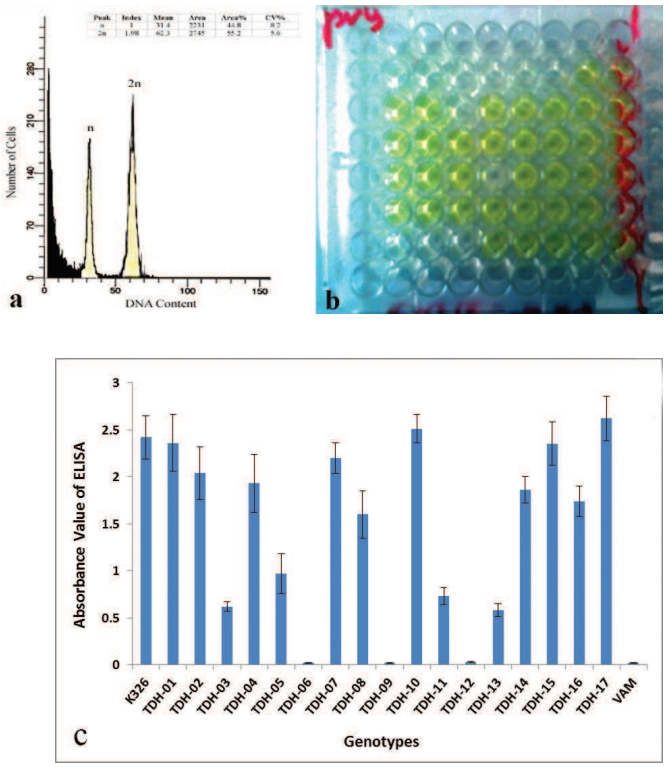

Fig. 4. (a) Flow cytometric histogram of DNA content in haploid (n) and doubled haploid (2n) plants of *N. tabacum* cv. VAM x K 326, (b) The results of DAS-ELISA test of 7 doubled haploids, cultivar K326 and negative control (NC) and (c) Absorbance values of A_{405} in DAS-ELISA test

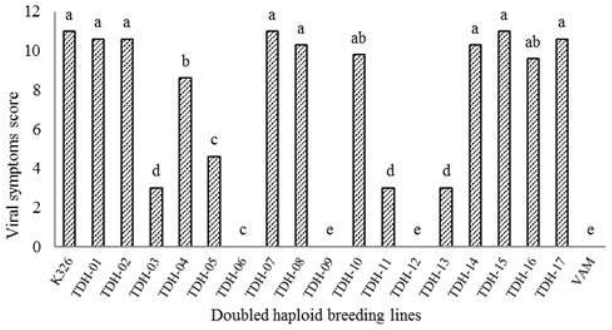

Fig. 5. Comparison of the resistance to PVY in doubled haploid lines (TDHs) and their parental forms: *N. tabacum* cv.VAM and K326. The columns with same letters, are not significantly different according to Duncan's Multiple Range Test

genotypes in respect of all the measured traits at 5 % level of probability. The length of leaves in TDH-12 was significantly higher than that in control cv. K 326. The width of leaves in TDH-05, TDH-06, TDH-11 and TDH-13 lines was significantly higher than that in

control. The stem diameter (SD) and fresh weight of leaves (FWL) were significantly highest in TDH-06, TDH-12 and TDH-13 lines. TDH-06 and TDH-09 had significantly higher dry weight of leaves (DWL) than the weight observed in control plants. There were statistically significant differences for sugar content among all of the genotypes except TDH-06 and TDH-12 (which had a lower content than control). The analysis for nicotine content revealed that TDH-03 and TDH-05 as well as control were in one group and other lines were in other groups with higher value (Table 2).

Final lines selection

GGE–biplot graph with two main components (PC1 and PC2; R^2 = 82.4 %) was applied in order to select the proper lines (Fig. 6). On the presented graph, seven

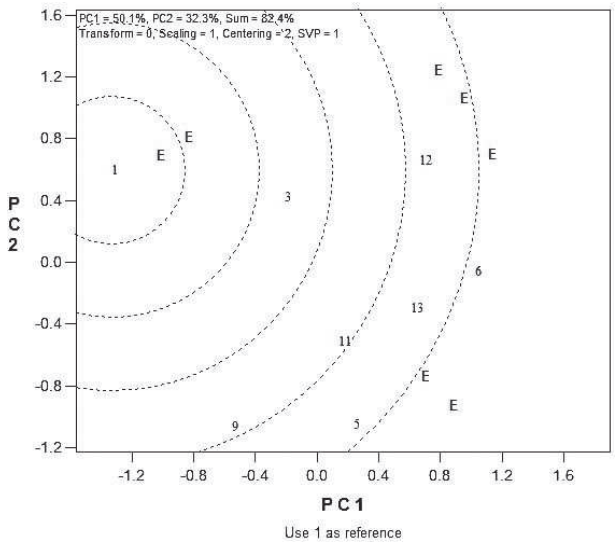

Fig. 6. Comparison of *N. tabacum* parental cultivar K326 (1) and doubled haploids lines based on the agronomic traits (E)

DH lines were compared with commercial cultivars K326 (No. 1) in respect of all studied traits. The circles indicate which of the tested lines is the most similar to the control cultivar which is located in the center. Based on the results, three DH lines named TDH-03, TDH-09 and TDH-11 were finally selected as *PVY* resistance with desirable traits (Fig. 7).

Discussion

The *va* allele has been introgressed into several tobacco genotypes by traditional plant breeding, such as in TN86 (Gupton 1980), or by *in vitro* regeneration of doubled haploids, such as in NC744 and NC745

Table 2. Details of agronomic and chemical traits in K326 and TDHs

Lines / Traits	LL†(cm)	LW(cm)	LN	HP(cm)	SD(mm)	FWL(Kg)	DWL(Kg)	SC(%)	NC(%)	SC/NC
K326	70.0±2.5^{b}	32.7±1.8^{b}	21.4±1.3^{a}	117.2±3.8^{bc}	30.7±1.9^{c}	61.7±3^{b}	8.9±1.2^{c}	15.6±1.7^{a}	2.3±0.9^{b}	6.7±1.5^{a}
TDH-03	74.3±2.5^{ab}	33.7±1.8^{b}	20.8±1.4^{a}	126.4±3.8^{ab}	32.3±1.9^{abc}	67.0±3^{ab}	9.8±1.3^{abc}	15.3±1.7^{a}	2.4±0.9^{b}	6.3±1.4^{a}
TDH-05	70.9±2.5^{ab}	37.6±1.8^{a}	20.9±1.3^{a}	128.4±3.8^{ab}	30.9±1.9^{bc}	65.4±3^{b}	9.1±1.2^{bc}	15.4±1.8^{a}	2.1±0.9^{b}	7.3±1.5^{a}
TDH-06	74.8±2.5^{ab}	37.6±1.8^{a}	20.2±1.4^{a}	119.2±3.8^{ab}	33.8±1.9^{ab}	76.1±3^{a}	10.6±1.3^{a}	12.0±1.7^{bc}	3.4±0.9^{a}	3.7±1.5^{b}
TDH-09	63.4±2.5^{c}	34.1±1.8^{b}	18.6±1.4^{a}	107.3±3.8^{c}	27.8±1.9^{d}	60.5±3^{b}	10.0±1.2^{ab}	13.0±1.6^{abc}	4.0±0.9^{a}	3.3±1.4^{b}
TDH-11	69.6±2.5^{d}	38.1±1.8^{a}	20.1±1.4^{a}	125.0±3.8^{ab}	31.6±1.9^{bc}	64.5±3^{b}	9.9±1.2^{abc}	14.7±1.7^{ab}	3.9±0.9^{a}	4.0±1.4^{b}
TDH-12	76.1±2.5^{a}	32.8±1.8^{b}	21.6±1.4^{a}	117.1±3.8^{bc}	35.0±1.9^{a}	76.6±3^{a}	9.4±1.2^{bc}	11.1±1.8^{c}	3.7±0.9^{a}	3.3±1.5^{b}
TDH-13	71.9±2.4^{ab}	37.6±1.7^{a}	20.3±1.4^{a}	129.4±3.8^{a}	32.8±1.8^{abc}	68.9±2.9^{ab}	9.8±1.2^{abc}	14.1±1.6^{ab}	3.2±0.9^{a}	4.5±1.4^{b}

†: LL = Leaf length, LW = Leaf width, LN = Leaf number, HP = Height of plant, SD = Stem diameter, FWL = Fresh weight of leaf, DWL = Dry weight of leaf, SC = Sugar content and NC = Nicotine content. Means in columns with the same letters, are not significantly different according to Duncan's Multiple Range Test

Fig. 7. Plant habit of doubled haploid lines obtained from *N. tabacum* cv. VAM x K 326

(Burk et al. 1979; Chaplin et al. 1980). Gooding and Kennedy (1985) reported that while cultivar VAM was completely resistant to PVY, cultivar NC744 facilitated the emergence of resistance-breaking variants. Thus, Kim et al. (2014) concluded that this phenomenon offers an opportunity to explore the basis of resistance durability and the evolution of viruses to overcome resistance. We observed tolerance diversity in our TDHs while all of them were homozygote (*vava* or *VaVa*) and must be resistant or susceptible. So there are possibly epistatic effects in *va* locus with other loci.

Overcoming PVY resistant *va* gene (isolate named PVY-ToBR1) was recently observed (Kim, et al. 2014; Lacroix, et al. 2011). Based on comparative sequence analysis between a resistance breaking isolate and a non-resistance breaking isolate, PVY-encoded VPg protein was identified as the determinant for overcoming VAM resistance (Masuta, et al. 1999). Recently, some molecular markers are available for PVY resistance in *Nicotiana tabacum* such as RAPD, AFLP and SCAR (Julio, et al. 2006; Tajima, et al. 2002). So in breeding for PVY resistance, it is better that use from this markers.

Quality in tobacco is most important aspect for cigarette production. Sugar and nicotine content are some of main factors for quality. High sugar content cause high quality leaf after curing and pleasant taste in cigarette and also high nicotine content cause unpleasant taste in cigarette (Davis and Nielsen 1999). We calculated the ratio of sugar/nicotine (SC/NC) (Table 2). Correlation between PVY symptoms and SC/NC was 0.91. This factor was 6.7±1.5 in K326, and in TDH-03 and TDH-05, it was near the K326 which does not show complete resistance to PVY^0 (Fig. 5). This ratio was under 3.7 in TDHs 06, 09 and 12 which had perfect resistance to PVY^0. These results showed that breeding for PVY resistance usually produces low quality tobacco. So breeder must be careful in this aspect, as well as in parent selection. Finally, a breeder must be able to equilibrate in quality, quantity and resistance. Sometimes it is necessary to waive from high-resistance for the benefit of quality or quantity.

Authors' contribution

Conceptualization of research (ZSM); Designing of the experiments (ZSM); Contribution of experimental materials (ZSM, AH); Execution of field/lab experiments and data collection (ZHM, AH); Analysis of data and interpretation (ASM, AH); Preparation of the manuscript (JS, ATG).

Declaration

The authors declare no conflict of interest.

References

Acosta-Leal R. and Xiong Z. 2008. Complementary functions of two recessive R-genes determine resistance durability of tobacco 'Virgin A Mutant' (VAM) to Potato virus Y. Virology, **379**: 275-283.

Bekkuzhina S. S. and Kalashnikova E. A. 2009. Selection

of wheat for resistance to the fungal pathogen Septoria nodorum Berk. In an isolated anther culture. Russ. Agricult. Sci.., **35**: 215-217.

Burk L. G., Chaplin J. F., Gooding G. V. and Powell N. T. 1979. Quantity production of anther-derived haploids from a multiple disease resistant tobacco hybrid. I. Frequency of plants with resistance or susceptibility to tobacco mosaic virus (TMV), potato virus Y (PVY), and root knot (RK). Euphytica, **28**: 201-208.

Chaplin J. F., Burk L. G., Gooding G. V. and Powell N. T. 1980. Registration of NC 744 Tobacco Germplasm1 (Reg. No. GP18). Crop Sci., **20**: 677-677.

Chawla H. S. 2003 Plant Biotechnology: A Practical Approach. Science Publishers.

Chidan Kumar C. S., Mythily R. and Chandraju S. 2012. Estimation of sugars by acid hydrolysis of paddy husk by standard methods. J. Chem. Pharma. Res., **4**: 3588-3591.

Ciuperca A., Daunescu A. D., Paunescu M., Burcea A. M. and Miltaru D. C. 2003. Research methods used in the release of tobacco cultivars, with double resistance to PVY and TMV in Romania. In: CORESTA Meeting Agro-Phyto Group, Bucharest, APOST 8.

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

Czubacka A., Doroszewska T. and Trojak-Goluch A. 2012. Agronomic characteristics of transgenic tobacco doubled haploids resistant to *Potato virus Y*. Food, Agric. Env., **10**: 374-378.

Davis D. L. and Nielsen M. T. 1999. Tobacco: Production Chemistry and Technology. Blackwell Science

Dhooghe E., Van Laere K., Eeckhaut T., Leus L. and Van Huylenbroeck J. 2011. Mitotic chromosome doubling of plant tissues in vitro. Plant Cell Tiss. Organ Cult., **104**: 359-373.

Doroszewska T. and Chrzanowska M. 2001. Characterization of the main PVY resistance sources to different PVY strains. In: CORESTA Meeting Agro-Phyto Group, Cape Town, South Africa, p. 5.

Gorrod J. W. and P. Jacob 1999. Analytical Determination of Nicotine and Related Compounds and their Metabolites. Elsevier Science.

Gupton C. L. 1980. Registration of Greeneville 107 Burley Tobacco Germplasm1 (Reg. No. GP17). Crop Sci., **20**: 116-116.

Julio E., Verrier J. L. and Dorlhac de Borne F. 2006. Development of SCAR markers linked to three disease resistances based on AFLP within *Nicotiana tabacum* L. Theor. Appl. Genet., **112**: 335-346.

Kim J.-H., Kim Y.-S., Jang S.-W. and Jeon Y.-H. 2014. Characterization of a novel resistance-breaking isolate of potato virus Y in *Nicotiana tabacum*, 3:10.

Koelle G. 1958. Ver suche zur Vererbung der Krankheitsresistenz bei Tabak. 2 Mitt. Eine Rippen-bräune-resistente Virgin A-Mutante nach Anwendung künstlicher Mutationsaulösungdurch Röntgenstrahlen. Tabak-Forschung, **24**: 83-84.

Lacroix C., Glais L., Verrier J. L., Charlier C., Lorencetti C. and Jacquot E. 2011. Impact of tobacco recessive resistance gene va on biological properties of Brazilian Potato virus Y (PVY) isolates. Plant Pathol., **60**: 1048-1054.

Masuta C., Nishimura M., Morishita H. and Hataya T. 1999. A single amino Acid change in viral genome-associated protein of potato virus y correlates with resistance breaking in 'virgin a mutant' tobacco. Phytopathol., **89**: 118-123.

Miller R. D. 1987. Registration of 'TN 86' Burley Tobacco. Crop Sci., **27**: 365-366.

Nakamura A., Takashima S., Hasegawa H. and Matsumoto T. 1993. Simple and Efficient Chromosome Doubling Method by Colchicine-treatment for Haploid Plantlets of Tobacco. Ikushugaku zasshi, **43**: 603-612.

Nichols W. A. and Rufty R. C. 1992. Anther culture as a probable source of resistance to tobacco black shank caused by Phytophthora parasitica var 'nicotianae'. Theor. Appl. Genet., **84**: 473-479.

Otto F. 1990. DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. Methods in Cell Biol., **33**: 105-110.

Patrascu M., Paunescu A. D. and Ciuperca A. 1999. Transfer of the resistance to TSWV from Nicotiana alata to some tobacco cultivars, using the *in vitro* culture technique. In: CORESTA Meet, Agro-Phyto Groups, Suzhou, POST8.

Pour Mohammadi P., Moieni A., Ebrahimi A. and Javidfar F. 2012. Doubled haploid plants following colchicine treatment of microspore-derived embryos of oilseed rape (*Brassica napus* L.). Plant Cell Tiss. Organ. Cult., **108**: 251-256.

Quenouille J., Vassilakos N. and Moury B. 2013. Potato virus Y: a major crop pathogen that has provided major insights into the evolution of viral pathogenicity. Molecular Plant Pathol., **14**: 439-452.

Rauf S., Khan I. A. and Khan F. A. 2006. Colchicine-induced tetraploidy and changes in allele frequencies in colchicine-treated populations of diploids assessed with RAPD markers in *Gossypium arboreum* L. Turkish J. Biol., **30**: 93-100.

Schiltz P. 1967. Creation de N. tabacum Resistants a P. tabacina. Analyse Histologique et Biologique de la Resistance. These de doctorat d'Etat, Universite de Bordeaux

Shahadati Moghaddam Z., Hossainzadeh F. N. and Zamani P. 2006. Effect of growth regulators in anther

culture of tobacco. In: The 9th Iranian Crop Sciences Congress, University of Tehran, p 381.

Smýkalová I., Šmirous P., Jr., Kubošiová M., Gasmanová N. and Griga M. 2009. Doubled haploid production via anther culture in annual, winter type of caraway (*Carum carvi* L.). Acta Physiol. Plant, **31**: 21-31.

Solov'eva L. V. 1990. Number of chloroplasts in guard cells of stomata as an indicator of the ploidy level of apple seedlings. Cytol. Genet., **24**: 1-4.

Sood S., Dhawan R., Singh K. and Bains N. S. 2003. Development of novel chromosome doubling strategies for wheat × maize system of wheat haploid production. Plant Breed., **122**: 493-496.

Tajima T., Noguchi S., Tanoue W., Negishi H., Nakakawaji T. and Ohno T. 2002. Background Selection Using DNA Markers in Backcross Breeding Program for Potato Virus Y Resistance of Tobacco. Breed. Sci., **52**: 253-257.

Takashima S., Hasegawa H. and Nakamura A. 1995. A Simple Method for Chromosome Doubling in Tobacco Anther Culture - Direct Application of Colchicine to Anthers before culture. Japanese J. Breed., **45**: 107-110.

Trojak-Goluch A. and Berbeæ A. 2011. Growth, development and chemical characteristics of tobacco lines carrying black root rot resistance derived from Nicotiana glauca (Grah.). Plant Breed., **130**: 92-95.

Verrier J. L., Marchand V., Cailleteau B. and Delon R. 2001. Chemical change and cigarette smoke mutagenicity increase associated with CMV-DTL and PVY-N infection in Burley tobcco. In: CORESTA Agronomy-Phytopathology Meeting, Cape Towne, South Africa.

Wenzel G. and Uhrig H. 1981. Breeding for nematode and virus resistance in potato via anther culture. Theor. Appl. Genet., **59**: 333-340.