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Positive Transgressive Segregation in Intervarietal Crosses of Tobacco (*Nicotiana tabacum* L.) and their Micropropagation

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ABSTRACT: Three advanced oriental tobacco (*Nicotiana tabacum* L.) lines, derived from cross between three commercial cultivars tobacco and selected by their positive transgressive traits, are described. *Transgressive* segregation by agronomically significant quantitative traits such as plant height, number of leaves, vegetation period (time to maturity) and yield/plant was observed. Content of nicotine, reducing sugars, and total nitrogen was determined in the leaves. Compared to the better parent, the yield per 1000 m^2 exceeded over 18.92% (Line 44), 30.81% (Line 58/2) and 56.76% (Line 59), respectively. In field conditions, the all three lines presented combined disease resistances to Tobacco Mosaic Virus (*TMV*), black shank disease (*Phitophtora parasitika var. nicotianae*) and downy mildew (*Peronospora tabacina*). Based on these results, we inferred that the transgressive selection can be used for development of valuable lines of interest of tobacco breeding. An effective micropropagation protocol of the transgressive tobacco lines was developed. As initial explants were used in vitro 30 days germinated seedling. The highest propagation rate was recorded on MS medium containing 1.0 mg L^{-1} 6-Benzylaminopurine (BAP) and 0.1 mg L^{-1} Indole-3-Acetic Acid (IAA) after three weeks of culture. The plants were successfully rooted in ½ MS medium containing 0.1 mg L^{-1} Indole-3-Butyric Acid (IBA). The developed efficient simple protocol in the study could be used for obtaining of plants for breeding programs of *N. tabacum*. The studied lines may be released as new varieties or used to produce improved tobacco hybrids.

Key words: Tobacco, hybrids, transgressive selection, yield, shoot multiplication.

INTRODUCTION

The tobacco plant is one of the most important commercial nonfood crops grown in the world today. Aromatic tobacco, often referred to as Turkish or oriental, is a sun-cured, highly-aromatic, small-leafed variety (*Nicotiana tabacum* L.) that is grown in Turkey, Greece, Bulgaria, and Macedonia. There is significant commercial expertise in tobacco farming that traditionally focused on biomass and alkaloid production mainly on nicotine concentration as the principal alkaloid (Djordjevic and Doran, 2009). Oriental tobacco is typically cultivated on low fertility soils without fertilization, and produces smaller leaves than flue-cured and Burley types. The yield, quality, and agricultural characteristics of tobacco are significantly influenced by environmental conditions as the different tobacco varieties exhibited specific sensibility to environment (Saitanis *et al.,* 2001), thus suggesting that the choice of the proper cultivars is a crucial factor for enhancement of the production level.

Tobacco crop is a regional culture with specific growing areas located mainly in southern Bulgaria. Over 20 years, tobacco varieties have been grown at multiple sites as part of international trade agreement following the requirement for producing homogeneous raw material. The principal varieties of the oriental type are Basma having fine flavor, pleasant taste and very good burning capacity. The climate of Bulgaria benefit tobacco seedbeds but during the last decade, the most economically important tobacco varieties were grown in unfavorable environmental conditions and therefore, they lost some valuable agronomic and chemical characteristics such as homogeneity, typically, chemical compounds, phenotypic expression etc.

Industrial tobacco breeding targets complex traits. Although there are significant commercial results, different new lines and cultivars are likely to be needed when the objective is to optimize biological expression, plasticity, quality and yield. Hybridization is a useful tool for creating genetic variation within the crop species to produce transgressive segregants. Along with conventional breeding methods, the production of extreme or transgressive phenotypes in segregating hybrid populations seems to be an alternative way for obtaining the new valuable plant materials. According to Riesenberg *et al.* (1999), transgressive segregation is the production of F_2 or later generation hybrid progeny with phenotypes that can fall outside the phenotypic range of the parental populations from which they were derived. Such plants are produced by the accumulation of favorable genes from both the parents as a consequence of recombination. It can be caused by genes with dominance or additive effects, by gene interactions such as epistasis, and by environmental effects. Transgressive phenotypes can be produced when alleles at multiple loci that originated in different parental populations recombine in the hybrids. This paradoxical expansion of phenotypic variation beyond the range of both parental populations depends upon the existence at some loci of alleles that increase the value of the phenotype and of alleles at other loci that reduce its value within each of the hybridizing parental populations. Transgressive segregation is very important in applied genetics (Yadav *et al*., 1998; Riesenberg *et al*., 1999; Migge *et al.,* 2000; Zhang *et al*., 2001; Toth *et al.,* 2003; Bell *et al.,* 2005; Kuczynska *et al.,* 2007; Ramya *et al.,* 2012). Various approaches and analysis have been applied to predicting the frequency of transgressive segregants in a plant population, although the prediction of transgressive segregation is still a problem (Rieseberg *et al.,* 2003; Kuczyńska *et al.,* 2007; Kirk *et al.,* 2012). It seems promising to apply an approach measuring the performance of the parental genotypes and estimating their genetic distance by molecular markers (Kuczyńska *et al.,* 2007).

In tobacco, transgressive segregation is not well studied but as a biological phenomenon it is important breeding opportunity for phenotypic innovation. It was established that the transgression frequency is higher in combined hybridization, especially in cases where the wild species is involved. Based on this approach, a number of oriental tobacco cultivars were developed. They all were characterized by higher number of leaves and longer vegetation period. Many authors suggested that the appearance and multiplication of transgressive phenotypes in advanced generations should lead to increased level of tobacco breeding (Didenko, 1974; Persesjan, 1987).

Tobacco breeding in Bulgaria can be regarded as part of the larger tobacco improvement effort in several European countries such as France, Greece, Croatia, Spain and some others. The current investigation is a portion of a tobacco research program with the objective of producing and evaluating new germplasm lines providing useful materials for plant breeding. Herewith, we examined and evaluated three new oriental tobacco breeding lines of segregated hybrids exhibiting positive transgression by several traits.

In vitro technique offers possibilities for mass propagation of economically important plant species. Moreover, in vitro propagation of tobacco lines and cultivars is important to support breeding programs. *N. tabacum* have been regenerated from a range of tissues, but the most commonly used are leaf explants. Earlier organogenesis via callus culture from leaf disc (Gowher *et al.,* 2007; Rahman *et al.,* 2010) and somatic embryogenesis (Pathi *et al.,* 2013) has been reported. However, these protocols involve several stages and require time. Here we report a very simple and effective protocol for in vitro propagation of *N. tabacum*. The aim of the present study was to describe and evaluate three new oriental tobacco lines derived after transgressive segregation and to establish of optimum culture conditions for their micro propagation.

MATERIALS and METHODS

A. Agricultural tools

Initial plant materials and phenotypic characterization. Three transgressive oriental tobacco lines № 58/2, № 44 and № 59 were included in the current investigation. They were developed after conventional combined crosses among three sub-group of commercial cultivar Krumovgrad: Krumovgrad 90, Krumovgrad 58, and Krumovgrad 988. The parents involved in hybridization are one of the most widespread oriental tobaccos cultivated in Bulgaria. Experiments were carried out in regional trials: in the Experimental Field of the Institute of Plant Physiology and Genetics, BAS, Sofia, Bulgaria in 2013-2015 years. Each trial was laid out in a randomized block design with two replications; each block with area 25 m^2. There were 15000 plants per 1000 m^2 (50/13 cm each plot). Cultivar Krumovgrad 90 (K 90) was used as the control. Tobacco production and field plot maintenance were performed according to tobacco production recommendations for Bulgaria. Standard agronomic practices for each specific region were applied. The transgressive lines were characterized at the phenotypic level. Observations such as plant height (cm), number of leaves, and vegetation period (time to maturity) were taken. After harvesting, plot yield per plant was determined and quality was assessed.

Chemical composition of tobacco lines. Percent nicotine and percent reducing sugars, and amount of total nitrogen in the leaves of tobacco were determined for the cured leaves in middle stock position (fifth and sixth reaping) on plot basis as follow: nicotine, % - according to ISO 15152 specifies a method for the determination of the content of total alkaloids as nicotine in tobacco by continuous-flow analysis; reducing sugars, % - according to ISO 15154 specifies a method for the determination of the content of reducing carbohydrates in tobacco by continuous-flow analysis; total nitrogen, % - Bulgarian State Standard 15836-88. Analyses were performed at the Tobacco and Tobacco Products Institute – Plovdiv. Each analysis was repeated two times in four samples.

In field conditions, the resistance to Tobacco Mosaic Virus (*TMV*), black shank disease (*Phytophthora parasitica var*. *nicotianae*) and downy mildew (*Peronospora tabacina*) was observed.

The level of transgression was calculated as follow:

$$Tr = \frac{F2.100}{Pb},$$

where F_2 was the value of the trait in F_2, and P_b - the value of the trait of the better parent.

B. Biotechnological tools

Experiments were carried out at the Laboratory of Plant Biotechnology of the Institute of Plant Physiology and Genetics, BAS, Sofia, Bulgaria in 2014-2015 years. Seeds from three transgressive oriental tobacco lines were used for in vitro germination. Surface sterilization was performed by immersion of the seeds in 70% (v/v) ethanol for 2 min, followed by 15 min in a 15% (v/v) sodium hypochlorite solution, followed by three rinses in sterile distilled water. Disinfected seeds were germinated aseptically in Petri dishes (d=10) containing 20 ml of Murashige & Skoog, 1962 (MS) medium with MS vitamins. In vitro grown seedlings (20 days after germination) were used as the source of primary explants. Shoot induction medium consisted of MS medium, 10 g L^{-1} sucrose, solidified with 0.8 g L^{-1} and supplemented with BAP (0, 0.5 and 1.0 mg L^{-1}) and low level of auxin IAA (0.1 mg L^{-1}). Newly formed shoots were excised after three weeks of culture and were transferred to half-strength MS medium without auxin and with 0.1 or 0.5 mg L^{-1} IBA for rooting of plants. Each treatment consisted of 20 replicates per treatment and the experiment was conducted twice. The plants with well-developed roots were transferred to plastic pots containing mix from soil, sand and perlite in volume 2:1:1 v/v/v for four weeks at ex vitro conditions.

All media were adjusted to pH 5.8 with 1N NaOH or HCl, prior to gelling with agar-agar and sterilized by autoclaving (121 °C for 20 min.). Cultures were maintained in growth room at 24±1 °C temperature, under 16/8 h light/dark regime, 40 μmol m^{-2} s^{-1} light intensity provided by white fluorescent light tube 36 W (Phillips).

C. Statistical analysis

Data were subjected to one-way ANOVA analysis of variance for comparison of means, and significant differences were calculated according to Fisher's least significance difference (LSD) test at the 5% significance level using a statistical software package (Statgraphics Plus, version 5.1 for Windows). Data were presented as means ± standard error.

RESULTS and DISCUSSION

A. Agricultural tools

Phenotypic variations in F_2 hybrid generation indicated that transgressive segregation occurred in segregating plants.

Some of the F_2 tobacco plants revealed an over-expression of agronomical traits exceeding the best phenotype of their varietal counterparts. In F_5 obtained after successive self-pollination, we selected the lines - segregants superior to parents with transgressive traits of interest for tobacco breeding - increased leaf number per plant or longer period of maturity. In the current study, we included three lines which are result from positive transgressive segregation in tobacco progeny developed after intraspecific crosses. Each of the breeding lines, grown under field conditions was easily recognized by its specific morphological and agricultural characteristics.

Tobacco breeding line 58/2. The plants were higher than the control cultivar - Krumovgrad 90 as the plant height reached 160-165 cm, thus exceeding the control with 26.9% (Fig. 1a). The leaves are shortly stalked, oblong-elliptical, shortly acuminate at the apex, decurrent at the base; they varied in size as the greatest one not exceeded 22/13 cm. Therefore, they could be classified as the first class of standard level (Fig. 2c, d). The transgression by number of leaves per plant reached 68.75% (50-52 leaves) (Fig. 1b). The vegetation period varied from 90 to 92 days (Fig. 1c). The yield sufficed for 242.5 kg/1000 m^2 which exceeded that of the higher parent (Fig. 1d). The nicotine concentration, sugar content and amount of nitrogen are given in Table 1.

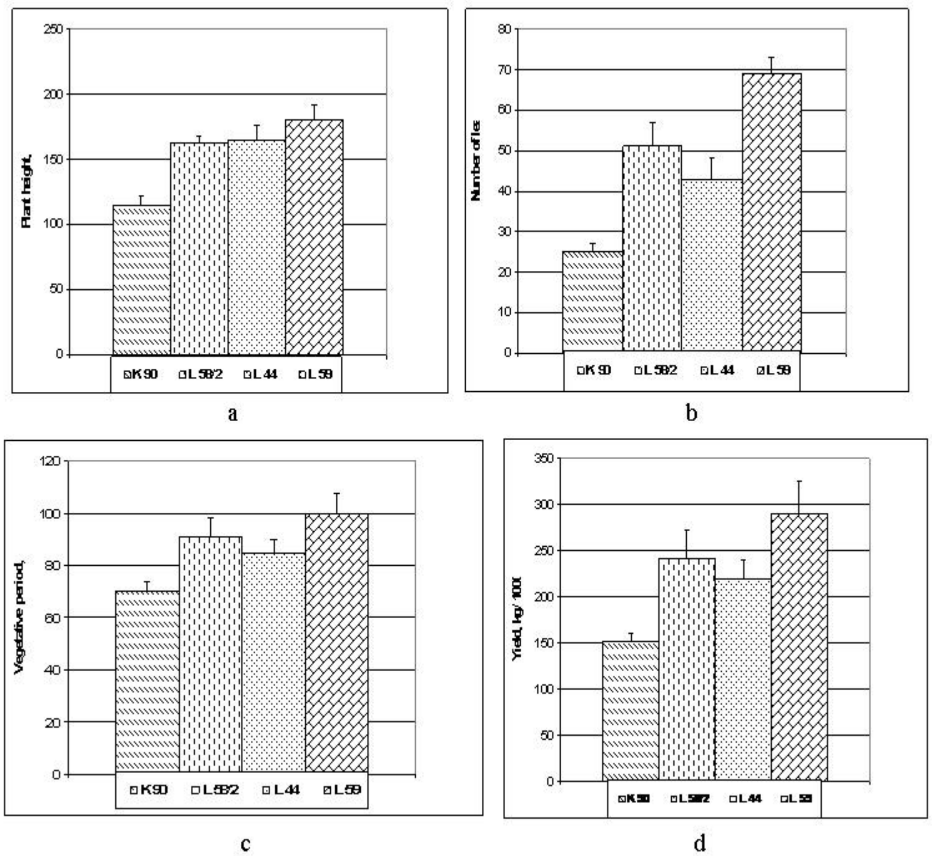

Fig. 1. a) Positive transgressions for plant height of three tobacco lines and control cv. Krumovgrad 90; b) Positive transgression for leaf number of three tobacco lines and cv. Krumovgrad 90; c) Positive transgressions for vegetation period of three tobacco lines and cv. Krumovgrad 90; d) Positive transgression for yield per plant of three tobacco lines and cv. Krumovgrad 90.

Tobacco breeding line 44. The line has pyramidal phenotype (Fig. 2e). The stem was right, stable and lodging resistant. The average plant height sufficed for 165 cm (Fig. 1a). The leaves were situated spirally as their number/plant varied from 42 to 44 technically usable (Fig. 2f). The level of positive transgression was 31.25% as shown in Fig. 1b.

The inflorescence is of middle size, rotund, slightly lifted from the upper leaf. All the plants flower simultaneously and very intensive. The vegetation period ranged from 84 to 86 days (Fig. 1c). The line 44 yielded 220 kg/1000 m^2 homogeneous, high-quality and resistant to burnt material, thus exceeding by yield the better parent with 18.92% (Fig. 1d). The dried detached tobacco leaves in the barn was typical for the sunny dried oriental tobacco, golden-yellow to orange with gentle and shiny foliage. The line contains 0.82% nicotine, 19.2% sugars and 1.6% total nitrogen (Table 1).

Tobacco breeding line 59. Morphologically, the plants were homogeneous with high productivity potential (Fig. 2g, h). The plant height sufficed for 180 cm (Fig. 1a), the number of leaves/plant - 68-70 (Fig. 1b), and vegetation period - 100 days (Fig. 1c), nicotine content - 1.13%, sugar content - 18.3% and total nitrogen content 1.81%. The level of positive transgression for plant height was 34.86%, for the number of leaves - 112.5%, and for vegetation period - 25%, respectively. The plants were characterized by well-developed vigorous roots with secondary branches (Fig. 3b); that exceed those of the parents which have 2 or 3 branches (Fig. 3a). The branches are in help for enhancement the water absorbing capacity facilitating the water supply to the plants. Thus, during growing period, the line 59 remained fresh for a long time with good turgor keeping the leaves from extended desiccation. As result, this is the line with the highest yield (290 kg/1000 m^2) that is 56.76% over the standard value (Fig. 1d).

Tobacco cultivar* Krumovgrad 90 *(control). The values of all examined parameters (plant height, number of leaves, vegetation period and yield) in plants of cultivar K 90 were lower compared to the transgressive lines (Fig. 2a, b). In chemical composition (nicotine, reducing sugars and total nitrogen) of the control K 90 and transgressive tobacco lines were not observed significant differences (Table 1).

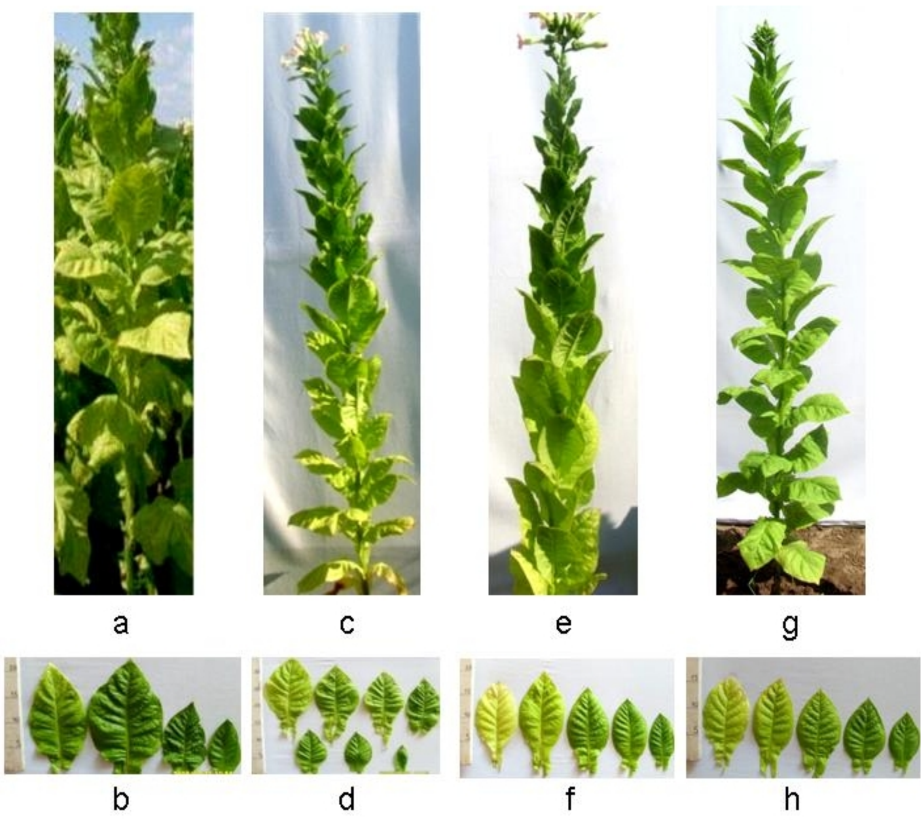

Fig. 2. Plants from transgressive tobacco lines: a) K 90; b) Leaves from the different plant zones of the K 90; c) Line 58/2; d) Leaves from the different plant zones of the L 58/2; e) Line 44; f) Leaves from different plant zones of the L 44; g) Line 59; h) Leaves from different plant zones of the L 59.

Table 1: Chemical characteristics of three transgressive oriental tobacco lines.

Tobacco lines	Nicotine, %	Reducing Sugars, %	Nitrogen, %
K 90	0.68±0.15 [a]	22.6±0.62 [b]	1.6±0.30 [a]
Line 58/2	0.68±0.12 [a]	22.8±0.65 [b]	1.8±0.34 [a]
Line 44	0.82±0.18 [ab]	19.2±0.52 [a]	1.6±0.28 [a]
Line 59	1.13±0.27 [b]	18.3±0.49 [a]	1.8±0.31 [a]

Data are presented as means of four replicates ± standard error. Different letters indicate significant differences assessed by the Fisher LSD test (5%) after performing ANOVA multifactor analysis.

a b

Fig. 3. Root system of transgressive tobacco lines: a) Roots from the K 90; b) Roots from the L 59.

The current results shows that the content of chemical substances in tobacco leaves were low as compared to the parental counterparts. Under field conditions, the lines were resistant to TMV, black shank disease caused by *Phytophthora parasitica var*. *nicotianae* and downy mildew (*Peronospora tabacina Adam Blue mold).* The use of transgression is a promising direction in the tobacco selection, although transgressive events in this species are not well understood. According to Dyulgerski *et al.* (2015) transgressive manifestations in the Burley tobacco hybrids in the second generation in terms of length of the vegetative period presented by degree and frequency are relatively low presented. The direction of crossing influences the degree and to a lesser extent the frequency of transgressive events in the length of the vegetation period in the studied hybrid combinations. Strong relationship between manifestations of heterosis and transgression has not been observed.

In general, information on agricultural characteristics and content of some important chemical substances (nicotine, reducing sugars and total nitrogen) demonstrated that the conventional breeding methods as combined hybridization (intercrosses between three or more cultivars) can generate transgressive hybrid phenotypes with extreme trait values exceeding the combined range of the parental species.

B. Biotechnological tools

The objective of first stage is to achieve an aseptic culture without contaminating bacteria or fungi. Sodium hypochlorite (15%) treatment for 15 minutes resulted in 100% clean cultures. The seeds from three trangressive tobacco lines were cultured on MS medium containing 1% sucrose and 0.8% agar, which allows successful germination after 20 days (Fig. 4a). The seedlings were sub cultured on the same medium twice, till the beginning of their stable growth and development (Fig. 4b). The tip explants from 20 days *in vitro* grown seedlings of *N. tabacum* were cultivated on MS medium supplemented with BAP at different concentrations in combination with low level of IAA for production of multiple vegetative shoots (Table 2). The shoot forming capacity was greatly influenced by the concentration of growth regulator in the medium. The shoots were differentiated from the base of the seedling. The addition of low level IAA to the medium containing BAP had stimulatory effect on the frequency of shoot induction and the shoot number per plant.

It was found that out of the three lines, L58/2 showed the highest micropropagation rate (100%) with mean number of shoots (10.2) on MS + 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} IAA after 3 weeks of culture (Figs. 4c and 4c[1]). The frequency (90%) with mean shoot number (6.5) showed L44 and the frequency (85%) with mean shoot number (6.1) showed L59 (Table 2). At higher concentrations of 1.5 mg L^{-1} BAP with 0.1 mg L^{-1} IAA, shoot number decreases significantly and exhibited reduced shoot development. The frequency of shoot multiplication was comparatively lower on this combination supplemented medium for the three tobacco lines. Several subcultures were carried out on the same optimum medium (MS + 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} IAA) for increased the number of shoots. This MS medium supported the better multiplication in terms of shoot propagation frequency, shoot number and shoot length.

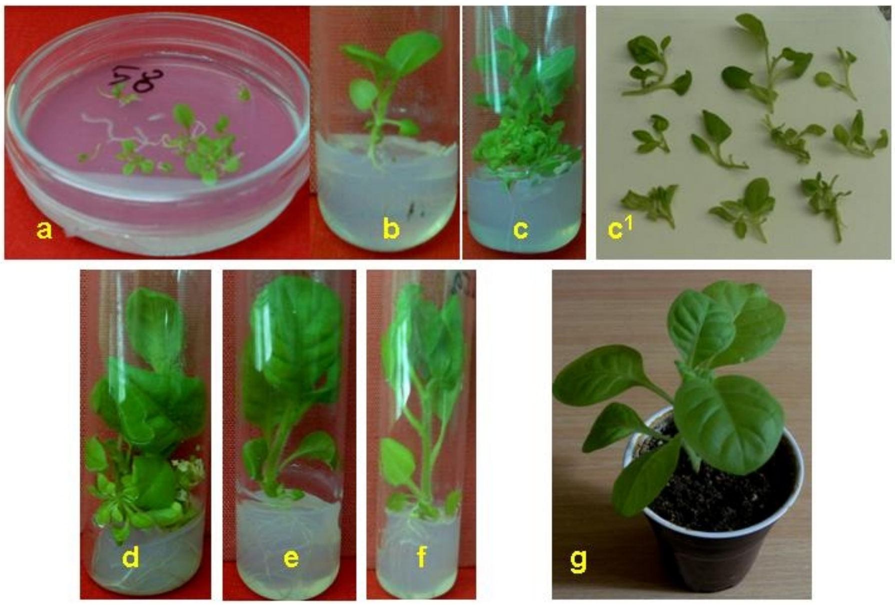

Fig. 4. Micropropagation of trangressive tobacco line 58/2: a) Seed germination on MS medium with vitamins; b) The seedling on MS basal medium; c) and c[1]) Multiple shoots on MS with 0.5 mg L^{-1} BAP + 0.1 mg L^{-1} IAA; d) Rooted plant on ½ MS without PGR; e) Rooted plant on ½ MS with 0.1 mg L^{-1} IBA; f) Rooted plant on ½ MS with 0.5 mg L^{-1} IBA and g) Ex vitro acclimatized plant.

Table 2: Effect of plant growth regulators (PGRs) on shoot induction of transgressive tobacco lines.

Plant growth regulator	Tobacco lines	Frequency of shoot induction, %	Number of shoots per explant	Shoot length, cm
MS without growth regulator	L44	45	1.2±0.11 [a]	2.1±0.19 [a]
	L58/2	55	1.8±0.16 [a]	2.9±0.26 [b]
	L59	40	1.7±0.15 [a]	3.0±0.29 [b]
MS + 0.5 mg L^{-1} BAP + 0.1 mg L^{-1} IAA	L44	85	4.2±0.35 [a]	3.1±0.30 [ab]
	L58/2	90	5.9±0.43 [b]	3.4±0.31 [ab]
	L59	80	5.4±0.45 [b]	4.3±0.40 [b]
MS + 1 mg L^{-1} BAP + 0.1 mg L^{-1} IAA	L44	90	6.5±0.48 [ab]	1.5±0.14 [a]
	L58/2	100	10.2±0.55 [c]	1.7±0.16 [a]
	L59	85	6.1±0.44 [ab]	1.6±0.15 [a]
MS + 1.5 mg L^{-1} BAP + 0.1 mg L^{-1} IAA	L44	60	3.8±0.31 [b]	1.2±0.08 [a]
	L58/2	70	4.3±0.34 [ab]	1.5±0.11 [a]
	L59	65	3.5±0.28 [b]	1.0±0.06 [a]

The data are presented as means of 20 plants per treatment ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing ANOVA multifactor analysis.

For the root induction, we establish previously that half strength MS medium with 0.1 or 0.5 mg L^{-1} IBA was found to be the best concentration for rhyzogenesis of several medicinal plants: *Arnica montana* (Petrova *et al.,* 2011), *Echinacea purpurea* (Zayova *et al.,* 2012), *Stevia rebaudiana* Bertoni (Zayova *et al.,* 2013) etc. In this study, root initiation began after 8 days of culture. The half-strength MS medium without auxin showed good root induction (Fig. 4d). Presence of IBA in ½ MS was found to be suitable for in vitro rooting of all studied lines. Maximum rooting was observed on the ½ MS medium supplemented with 0.1 mg L^{-1} IBA.

The highest mean number of roots 4.4 with a mean root length of 3.5 cm was developed from each plant for line 58/2 within 3 weeks (Table 3; Fig. 4e). Good rooting was observed on the ½ MS medium with 0.5 mg L^{-1} IBA (Fig. 4f). Auxin IBA has been reported to have stimulatory effect on tobacco root induction (Rahman *et al.,* 2010). The rooted plants of the tobacco lines were carefully removed from the in vitro to ex vitro conditions. They were transferred to plastic pots containing soil, perlite and sand in volume 2:1:1 (v/v/v) and successfully acclimatization in ex vitro conditions (Fig. 4g). About 90-95% of the propagated plants of all lines could survive under ex vitro conditions.

Table 3: Effect of IBA on root formation from *in vitro* multiple plants of transgressive tobacco lines.

Concentration of IBA	Tobacco lines	Rooted plants, %	Mean number of roots per plant	Mean length of roots, cm
½ MS control	L44	55	2.3±0.21 [a]	2.2±0.19 [a]
	L58/2	65	2.9±0.32 [b]	2.1±0.18 [a]
	L59	50	2.5±0.22 [a]	2.5±0.22 [b]
½ MS + 0.1 mg L^{-1}	L44	95	3.1±0.34 [ab]	3.3±0.26 [ab]
	L58/2	100	4.4±0.43 [c]	3.5±0.31 [ab]
	L59	95	3.5±0.36 [ab]	2.4±0.23 [b]
½ MS + 0.5 mg L^{-1}	L44	85	1.5±0.13 [a]	2.1±0.17 [a]
	L58/2	80	1.7±0.10 [a]	2.6±0.24 [b]
	L59	75	1.4±0.12 [a]	2.2±0.21 [a]

The data are presented as means of 20 plants per treatment ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing ANOVA multifactor analysis.

CONCLUSION

Transgressive segregation towards plant height, number of leaves, vegetation period and yield/plant indicated that different genes responding for these traits from different sources show additive effects. Thus, we should be able to develop new agronomical important genotypes in tobacco be intercrossing diverse cultivars. For example, tobacco breeding line 59 might be useful as breeding material for increasing percent nicotine since it exhibited the highest percentage for this alkaloid. The pyramidal phenotype of breeding line 44 suggests the potential value of this germplasm as a donor of alleles affecting lodging resistance, a character which is important in tobacco manufacturing. For yield, cured leaf quality, and other investigated agronomic traits, the described breeding lines were generally superior to the commercial cultivar Krumovgrad 90. Keeping in view the importance of tobacco, the present experiment proved that practical application of transgressive selection will be able to provide clues for development and introduction of new valuable tobacco lines and cultivars. This report provides a simple effective protocol for the mass propagation of plants from the tobacco lines by in vitro culture technique and long-term maintenance valuable plant material in the aseptic conditions for breeding program. The combination of cytokinin BAP (1.0 mg L^{-1}) and auxin IAA (0.1 mg L^{-1}) supplemented to MS medium was optimum for shoot induction. The rapid multiplication of *N. tabacum* allows breeders to introduce new lines and cultivars much earlier than they could by using conventional propagation techniques.

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