

Relationships among androgenetic components in wheat and their responses to the environment

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The effect of two nutrient media, Potato-2 and N6, on androgenetic component traits and their relationships were studied in wheat anther culture. Six wheat (*Triticum aestivum* L.) F₁ combinations (NS 20 × Driada, MV 05-2001 × Lana, MV 05-2001 × Zitnica, Driada × NS 403/00, Driada × Dacia and Odeska 162 × NS KV 124/01A) were used as material for anther isolation. Variations, interrelationships and path analysis for five androgenetic component traits (androgenetic capacity: AC, embryoid induction: EI, regeneration ability: RA, frequency of green plants: FGP and frequency of albino plants: FAP) were established. The average values for AC and EI were significantly lower on N6 medium, while for RA, FGP and FAP there were no significant differences between the Potato-2 and N6 media. Correlation coefficient analyses showed positive correlation between AC and EI ($r = 0.99^*$ on Potato-2 and $r = 0.86^*$ on N6), and between RA and FGP ($r = 0.95^*$ on Potato-2, $r = 0.93^*$ on N6), on both media. Additionally, the direct effects of RA and FAP on FGP were highly significant on both media, as determined by path coefficient analysis. A trait-association using environment biplot was applied to understand the environmental effects on these relationships. The biplot showed that anther culture trait associations were differently responsive to the environment.

Key words: wheat, anther culture, correlations, Path analysis, PCA.

INTRODUCTION

Specialized plant tissue culture methods have enabled the production of completely homozygous lines from gametic cells in a shortened time, compared to conventional plant breeding. Plants derived from gametic cells represent a homozygous array, each having a different genetic contribution from the parents. Doubled haploid production is widely used not only for plant breeding (De Buyser *et al.*, 1987; Pauk *et al.*, 1995), but also for basic research (Orshinsky *et al.*, 1990), such as genomic mapping, haploid transformation and artificial seed production (Tuvešson *et al.*, 2003).

Androgenesis is a common methodology used to develop haploids, and doubled haploids, in major

grain crops. The formation of androgenetic structures and regenerant plants depends on the genotype of the anther donor plant, its growth environment, culture conditions and their interactions (Lazar *et al.*, 1985; Redha & Talaat, 2008). Three parameters are usually used to estimate wheat androgenetic response: calli induction, regeneration and green plant percentage (Larsen *et al.*, 1991). These three androgenetic component traits have been found to be polygenically controlled and are independently inherited traits (Ekiz & Konzak, 1994). Their inheritance has been reported to be complex and controlled by different genetic mechanisms (He *et al.*, 1997; Schlegel *et al.*, 2000; Belinskaya, 2008).

Negative correlations among androgenetic component traits are frequently observed (Torp *et al.*, 2001; Chaudhary *et al.*, 2003). However, the environment may play an important role in the correlation between two traits, moderating their relationship

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(Yan *et al.*, 2007). This fits with the common understanding that the correlation between two quantitative traits is not fixed, rather, it is dependent on the genotypes that are tested and the environments in which they are tested (Yan & Wallace, 1995).

In the present study, relationships among parameters of androgenetic response in six wheat genotypes were tested by correlation and Path coefficient analysis. In order to establish the impact of environment on the relationships between androgenetic component traits, the study was conducted using two different induction media.

MATERIALS AND METHODS

In order to study androgenetic and regeneration abilities, six F_1 wheat (*Triticum aestivum* L.) combinations were selected: NS 20 × Driada, MV 05-2001 × Lana, MV 05-2001 × Zitnica, Driada × NS 403/00, Driada × Dacia and Odeska 162 × NS KV 124/01A. The combinations were created in the wheat breeding program at the Small Grains Department, at the Institute of Field and Vegetable Crops in Novi Sad, Serbia. Donor plants were grown under field conditions. Ten primary tillers from each combination were selected. Intact spikes were placed in jars with water, wrapped with foil and subjected to cold pre-treatment at 4 °C for 5-10 days. Pre-treated tillers were surface sterilized in 1.4% NaOCl for 5 min and rinsed five times with sterile distilled water. Only the middle portion of each spike, which contained anthers with microspores at the mid- to late- uninucleate stage, was retained for anther culture.

The effect of two induction media, Potato-2 (Chuang *et al.*, 1978), and N6 (Chu *et al.*, 1975) were compared in this study. The media contained different mineral salts and growth regulators. Composition of Potato-2 (P2) induction medium was: KNO_3 1000 mg l^{-1} ; $(\text{NH}_4)_2\text{SO}_4$ 100 mg l^{-1} ; $\text{Ca}(\text{NO}_3)_2 \times 4\text{H}_2\text{O}$ 100 mg l^{-1} ; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 125 mg l^{-1} ; KCl 35 mg l^{-1} ; KH_2PO_4 200 mg l^{-1} ; $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ 27.8 mg l^{-1} ; $\text{Na}_2 \times \text{EDTA} \times 2\text{H}_2\text{O}$ 37.3 mg l^{-1} ; thiamine 1.0 mg l^{-1} ; 2,4-D 1.5 mg l^{-1} ; kinetin 0.5 mg l^{-1} and 10% of extracts from fresh potato. In comparison with P2 medium, N6 contained higher concentrations (in mg l^{-1}) of KNO_3 (2830), $(\text{NH}_4)_2\text{SO}_4$ (463), $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ (185), KH_2PO_4 (400), the same amount of $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ (27.8) and $\text{Na}_2 \times \text{EDTA} \times 2\text{H}_2\text{O}$ (37.3), while $\text{Ca}(\text{NO}_3)_2 \times 4\text{H}_2\text{O}$ and KCl were completely omitted. Composition of N6 medium also included some compounds which were not present in P2 medium (in mg l^{-1}): $\text{CaCl}_2 \times$

H_2O (166), $\text{MnSO}_4 \times 4\text{H}_2\text{O}$ (4.4), $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ (1.5), H_3BO_3 (1.6) and KI (0.8). Besides the same concentration of thiamine (1.0 mg l^{-1}), N6 medium contained different organic compounds than P2 medium (in mg l^{-1}): pyridoxine (0.5), nicotinic acid (0.5), glycine (2.0) and myo-inositol (100). Levels of sucrose also were different in the media; P2 medium contained 9% sucrose while N6 medium contained 5% sucrose. The media organic components were sterilised with a cellulose membrane filter (pore size 0.22 μm) and added to autoclaved mineral salts solutions. Both media were agar-solidified (5%) and pH was adjusted to 6. The induction media were poured into 25 × 100 mm tubes, 25 ml of medium in each tube.

Anthers were isolated under aseptic conditions and placed onto induction medium. From each parental combination 540 anthers were isolated; half of the isolated anthers of each spike were cultivated on P2 medium and the other half on N6 medium. Thirty anthers were cultivated in each tube. Anther induction was carried out at 28-30 °C in the dark for 4-6 weeks. Embryogenic structures were then transferred to modified 190-2 regeneration medium (Zhuang & Jia, 1980) for plant regeneration. The cultures were maintained at 25-27 °C at a light intensity of 60-70 $\mu\text{mol m}^{-2} \text{s}^{-1}$, with photoperiod of 14/10 h.

The traits studied in this experiment were: androgenetic capacity (AC-number of responding anthers per 100 isolated anthers), embryoid induction (EI-number of embryoids formed per 100 isolated anthers), regeneration ability (RA – number of regenerated plants, both green and albino, per 100 calluses), frequency of green plants (FGP – number of regenerated green plants per 100 calluses) and frequency of albino plants (FAP – number of regenerated albino plants per 100 calluses).

The experimental design was a randomized complete block with three replicates. To provide sufficient, valid data for analysis, three tubes were used for each replication. Relationships between every pair of androgenetic traits were estimated by Pearson's simple correlation coefficients. Individual values of correlation coefficients of each medium were used for Principal Component Analysis (PCA) and plotted on the two-dimensional biplot (Gabriel, 1971). Direct and indirect effects of the androgenetic component traits on frequency of green plants were determined by the path-coefficient analysis (Li, 1975). Statistical calculations were done using the STATISTICA 7.1 program.

RESULTS AND DISCUSSION

Genotypic anther culture response

Data obtained with respect to androgenetic capacity, embryoid, green and albino plantlet development were recorded on a percentage basis. Arcsine transformation was carried out to standardize/normalize the data before the analysis of variance (Factorial ANOVA). The main effects of the genotype, medium and their interaction were all significant at the $p < 0.01$ level, for all androgenetic traits (Table 1).

The androgenetic response of six wheat F_1 combinations on different induction media (P2 and N6) are shown in Table 2. The androgenetic capacity (AC) on P2 medium varied from 3.5 to 5.9%, with average value of 4.7%, while on N6 medium, the variation was from 2.4 to 3.9%, averaging 3.0%. In general, AC and embryoid induction (EI) were improved with P2, with five of the six wheat F_1 combinations having significantly higher AC and EI results on P2 medium. For one combination (NS 20-05 \times Driada) however, there

were no significant differences between the media in terms of AC or EI results. The ability to regenerate plants (RA) was significantly affected by the medium in some combinations, but not others. Two combinations, Driada \times NS 40S-00 and Odeska 162 \times NS KV 124-1A, yielded significantly more plants on P2 medium, while another combination, MV 05-2001 \times Žitnica, yielded significantly more plants on N6 medium. The RA of the remaining three combinations was unaffected by the media and showed no significant differences between either medium.

The results also indicate a relationship between significant improvements in RA leading to significant improvements in both numbers of green and albino plants. For the cross, MV 05-2001 \times Žitnica, there was significantly better RA on N6 medium and this cross also had significantly higher numbers of green and albino plants on the same medium (N6). Two other crosses (Driada \times NS 40S-00 and Odeska 162 \times NS KV 124-1A) had significantly better RA on P2,

TABLE 1. Mean squares for Analysis of Variances of the androgenetic component traits

Source of variation	Androgenetic component traits ^b					
	df ^a	AC	EI	RA	FGP	FAP
Genotype (G)	5	4.6**	8.8**	57.30**	61.69**	95.298**
Medium (M)	1	110.8**	115.9**	12.65**	9.71**	6.780**
Interaction G \times M	5	10.1**	22.0**	182.70**	91.50**	217.396**
Error	24	1.1	0.3	0.15	0.19	0.692

^a degrees of freedom

^b AC: androgenetic capacity, EI: embryoid induction, RA: regeneration ability, FGP: frequency of green plants, FAP: frequency of albino plants

TABLE 2. Androgenetic response of six wheat F_1 combinations at different media (P2 and N6). Abbreviations are: AC: androgenetic capacity, EI: embryoid induction, RA: regeneration ability, FGP: frequency of green plants, FAP: frequency of albino plants

Genotype	AC		EI		RA		FGP		FAP	
	P2	N6	P2	N6	P2	N6	P2	N6	P2	N6
NS 20-05 \times Driada	4.1	3.9	4.4	5.2	21.1	28.6	21.1	21.4	0	7.2
MV 05-2001 \times Lana	5.6	3.6	6.3	4.0	22.6	29.8	19.0	26.2	3.6	3.6
MV 05-2001 \times Žitnica	5.2	3.2	5.6	3.5	25.6	40.4	25.6	36.6	0	3.9
Driada \times NS 40S-00	5.9	2.8	7.0	3.2	46.7	22.0	33.2	17.4	13.6	4.6
Driada \times Dačica	3.5	2.4	11.9	3.0	22.0	29.2	19.0	23.6	3.0	5.6
Odeska 162 \times NS KV 124-1A	4.1	2.4	4.4	3.5	49.3	23.8	41.5	23.8	7.8	0
Mean	4.7	3.0	6.6	3.7	31.2	29.0	26.6	24.8	4.7	4.1
LSD _{0.05}	0.6302		0.8202		7.6451		2.4937		1.2851	

which also flows onto significantly more green and albino plants on P2 medium. Other authors also found that some treatments which increases green plant production also increases the number of albino plants (Moieni *et al.*, 1997; Savaskan *et al.*, 1999; Grauda *et al.*, 2010).

The differences in the genotype's reactions to the induction media indicate that formation of androgenetic structures and plant regenerants (green and albino) depends on the genotype and the culture conditions which have also been established by other authors (Lazar *et al.*, 1985; Belinskaya *et al.* 2008; Khiabani *et al.*, 2008).

Correlation analysis

The Pearson correlation analysis (Table 3), for both media, showed that AC had a highly significant relationship with EI ($r = 0.99^*$ on P2, $r = 0.86^*$ on N6). These results are in agreement with results of Kondic (1999), Yildirim *et al.* (2008) and Ljevnaic (2009), in which highly significant correlations were found between AC and callus yield ($r = 0.98^{**}$, 0.833^{**} and 0.91^{**} , respectively). Androgenetic capacity, as well as EI had no significant correlations with other studied traits, on either media. There were non-significant negative correlations between AC and FGP ($r = -0.45$) and between EI and FGP ($r = -0.43$) on P2 medium. On N6 medium, there were positive non-significant correlations between FAP and AC ($r = 0.52$), and FAP and EI ($r = 0.42$). In contrast to our results, Belchev *et al.* (2000) found that in most cases high EI was connected with high GPY. Shlegel *et al.* (2000) also found that green and albino plant production were significantly positive correlated with callus induction. Based on these data, they concluded that the callus induction was the primary characteristic giving rise to green and albino production.

TABLE 3. Pearson's correlation coefficients (r) between tissue culture traits on P2 medium (above diagonal) and N6 medium (below diagonal). Abbreviations are: AC: androgenetic capacity, EI: embryoid induction, RA: regeneration ability, FGP: frequency of green plants, FAP: frequency of albino plants

Trait	AC	EI	RA	FGP	FAP
AC	1	0.99*	-0.29	-0.45	0.06
EI	0.86*	1	-0.28	-0.43	0.05
RA	0.33	0.08	1	0.95*	0.85*
FGP	0.13	-0.08	0.93*	1	0.66
FAP	0.52	0.42	0.17	-0.20	1

Highly significant correlations were found between regeneration ability and the number of green plants (Table 3), on both P2 and N6 media ($r = 0.95^*$ on P2, $r = 0.93^*$ on N6). A significant relationship between these two traits was also found by Tuvevsson *et al.* (1989). Given that RA is the total number of plants, it seems obvious that the number of green and albino plants will be correlated with this trait. However, results from a previous study (Kondic, 1999), as well as the results obtained by Ljevnaic (2009), showed a low correlation between regeneration ability and the number of green plants. The difference in the obtained results could be explained by different calculations for RA in these studies. In contrast to the present study, where RA, FGP and FAP were all calculated in relation to the number of calluses formed, in other mentioned studies (Kondic, 1999; Ljevnaic, 2009) RA was calculated in relation to the number of isolated anthers, while FGP and FAP were calculated in relation to the number of calluses formed.

A significant correlation ($r = 0.85^*$) was found between regeneration ability and the number of albino plants on P2 medium, but on N6 medium, the correlation between these traits was not significant ($r = 0.17$) (Table 3). These results are partly in agreement with those of Ljevnaic (2009) who studied the androgenetic ability of 24 genotypes of wheat and found, highly significant correlations between regeneration ability and androgenetic capacity ($r = 0.610^{**}$), and regeneration ability and callus yield ($r = 0.730^{**}$), but low correlation between regeneration ability and the number of albino plants ($r = 0.290$).

In another study on relationships between anther culture traits, Yildirim *et al.* (2008) found highly significant correlations between almost all traits studied, except between androgenetic capacity and number of albino plants, where they found only medium correlations. Negative correlations between tissue culture traits have been found by Larsen *et al.* (1991), Bruins & Snijders (1995) and Torp *et al.* (2001). The differences in relationships among androgenetic traits in different studies could be explained by differences in genetic material, the number of genotypes analyzed, environmental conditions, as well as differences in the definition and calculation of component traits.

In the last decade, studies about the connections and correlations between agronomic and tissue culture traits have received a lot of attention. Haliloglu *et al.* (2005) reported that the number of regenerated plants was positively correlated with the number of kernels per spike and grain hardness, but negatively

correlated with the number of spikes per m². An earlier study, conducted by Li *et al.* (2003), confirmed a positive correlation between callus formation and both kernel number per spike, and effective spikelets per spike. In comparison with the studies of Li *et al.* (2003) and Haliloglu *et al.* (2005), Dodig *et al.* (2008) found additional significant correlations with agronomic traits for each tissue culture trait. Grain yield showed the highest positive direct effect to number of calluses, while the highest impact on regeneration capacity was found for number of kernels per spike. However, they concluded that none of agronomic traits showed a strong association with any of tissue culture traits by a simple correlation analysis.

Environmental effects on relationships between anther culture traits

All ten anther culture trait associations responded differently to the media (Fig. 1). The biplot shows that the ten pair-wise associations fell into four groups. The first group included strong positive associations of AC versus EI, and RA versus FGP, on each media. Since these associations were highly significant on both media they can be regarded as very stable across environments. The second group was characterized by negative or insignificant associations on both media (EI versus RA, AC versus FGP and EI versus FGP), which could be characterized as relatively stable across environments. The third group consisted of AC versus RA, AC versus FAP and EI versus FAP which were positive and moderately strong on N6 medium, and very small, or negative, on P2 medium. The fourth group included RA versus FAP and FGP versus FAP which were strongly positive correlated on P2 medium, and insignificant on N6 medium. The differences between the associations from the third and the fourth groups on the media indicated that they were not stable across environments.

The results indicate that the most of the pairwise associations were responsive, to different extents, to the environment. This is in agreement with the results of other studies (Yan *et al.*, 2007; Dodig *et al.*, 2008; Gorjanovic *et al.*, 2010).

Path coefficient analysis

Correlations among traits were further analyzed using the path-coefficient approach, which involves partitioning the correlation coefficients into its components, direct and indirect ones through alternative traits or pathways (Dewey & Lu, 1959). FGP, as a com-

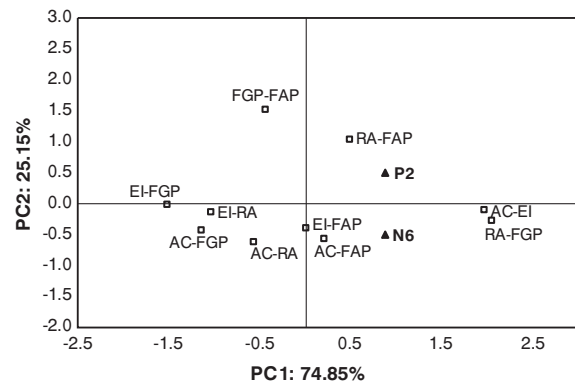


FIG. 1. A trait association by environment biplot, based on experiment with 6 wheat genotypes on two different media (P2 and N6). Abbreviations are: AC: androgenetic capacity, EI: embryoid induction, FGP: frequency of green plants, FAP: frequency of albino plants, RA: regeneration ability.

plex trait and the best estimate of the genotype's androgenetic potential, was viewed as the dependent variable, while AC, EI, RA and FAP were the independent variables.

This kind of approach provides a different picture to correlation analysis, because the path coefficient analysis can substantiate the findings of correlation analysis by providing a holistic view of trait associations which would not have been obtained from the correlation coefficients (Babar *et al.*, 2007; Marjanovic-Jeromela *et al.*, 2008).

The direct and the indirect effects of the androgenetic traits on FGP on two different induction media are shown in Table 4. Estimated correlation coefficients were highly significant on both media only between FGP and RA ($r = 0.930^{**}$ on P2 medium, $r = 0.950^{**}$ on N6 medium). The direct effects indicated that FGP was most dependent upon RA ($p_3 = 1.370^{**}$ on P2 medium, $p_3 = 0.996^{**}$ on N6 medium). The indirect effects of FAP on P2 medium was high and negative (-0.427), while other traits had almost no indirect effect on FGP. Highly significant, direct effects were also found for the number of albino plants ($p_4 = -0.503^{**}$ on P2 medium, $p_4 = -0.364^{**}$ on N6 medium). It was indirectly realized via RA (1.165) on P2 medium, while on N6 medium, the indirect effect of RA (0.169) and other traits was small. The androgenetic capacity and embryoid induction did not show significant direct or indirect effects on FGP, on either media.

The results of the present study demonstrate the strong influence of genotype and induction medium on a number of androgenetic traits in wheat. The correlation and path analysis demonstrate that the fre-

TABLE 4. Path coefficient analysis between frequency of green plants and other androgenetic traits on two induction media (P2 and N6). Abbreviations are: AC: androgenetic capacity, EI: embryoid induction, RA: regeneration ability, FGP: frequency of green plants, FAP: frequency of albino plants

Medium	Trait	P2					r ^a
		Direct effect	Indirect effect via				
			AC	EI	RA	FAP	
P2	AC	-0.090	—	0.068	-0.397	-0.030	-0.450
	EI	0.068	-0.089	—	-0.384	-0.025	-0.430
	RA	1.370**	0.026	-0.019	—	-0.427	0.930**
	FAP	-0.503**	-0.005	0.003	1.165	—	0.66
N6	AC	-0.013	—	0.004	0.329	-0.189	0.130
	EI	0.005	-0.011	—	0.080	-0.153	-0.070
	RA	0.996**	-0.004	0.000	—	-0.062	0.950**
	FAP	-0.364**	-0.007	0.002	0.169	—	-0.200

^a: Correlation coefficient; Determination coefficient: R² = 0.981** (P2), R² = 0.997** (N6)

quency of green plants was associated with the characters, RA and FAB, while there were no direct or indirect associations of FGP with AC and EI. These results are similar to other studies, where it has been found that the formation of the androgenetic structures and regenerant plants are under different genetic control mechanisms and that they are probably independently inherited (Ekiz & Konzak, 1994; He *et al.*, 1997; Schlegel *et al.*, 2000). Belinskaya (2008) also reported that androgenetic potential in spring barley is determined by several genes and quantitative trait loci (QTLs) with different chromosomal locations.

In order to overcome the problem of estimating the androgenetic potential of a large number of genotypes usually involved in breeding programs, there are two possible strategies for further studies. Based on the finding that there is some correlation between agronomic traits and tissue culture characteristics (Li *et al.*, 2003; Halilogu *et al.*, 2005; Dodig *et al.*, 2008), it could be useful to screen genotypes in field conditions and, by using established significant correlations as criteria, choose genotypes with potentially high androgenetic responses. However, this strategy could be limited by the fact that almost all significant correlations between field and tissue culture traits are environmentally dependent (Dodig *et al.*, 2008). Another strategy could involve the use of QTL mapping technology, which provides the possibility to estimate androgenetic potential at the molecular level. Future studies could involve gene mapping and the development of molecular markers for QTLs with a high potential for *in vitro* androgenesis.

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