Morphogenetic responses of embryo culture of wheat related to environment culture conditions of the explant donor plant

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ABSTRACT: Availability of immature embryos as explants to establish wheat (*Triticum aestivum* L.) by tissue culture can be limited by climatic factors and the lack of high quality embryos frequently hampers experimentation. This study evaluates the effects of rainfall, various temperature-based variables and sunshine duration on tissue culture response (TCR) traits including callus formation (CF), regenerating calli (RC), and number of plants *per* embryo (PPE) for 96 wheat genotypes of worldwide origin. The objectives of this study were to evaluate the significance of a particular climatic factor on TCR traits and to determine the period of wheat growth during which these factors were the most effective. The genotypes were grown in an experimental field during three seasons differing in meteorological conditions. The relationships between TCR traits and climatic factors within three time periods of wheat growth: 2, 6 and 10 weeks prior to embryo sampling were analysed by biplot analysis. The tissue culture traits were influenced at very different degrees by climatic factors: from 16.8% (RC) to 69.8% (CF). Donor plant environment with high temperatures and low rainfalls reduced ($p < 0.05$) the tissue culture performance of wheat genotypes. Callus formation was most sensitive to the temperature based factors. The environmental conditions between flowering and the medium milk stage were the most important for CF, while RC and PPE were not particularly related to any period.
Key words: *Triticum aestivum* L., biplot, correlation analysis, environment variable, growth period

Respostas morfogenéticas de embriões de trigo em função do ambiente de cultivo da planta doadora de explantes

RESUMO: A disponibilidade de embriões imaturos para estabelecer plantas de trigo (*Triticum aestivum* L.) por cultura de tecido pode ser limitada por fatores climáticos, e a falta de embriões de alta qualidade frequentemente dificulta a experimentação. Avaliou-se o efeito da chuva, de variáveis baseadas em temperatura e duração do brilho solar na resposta da cultura de tecido (RCT), incluindo a formação de calos (FC), regeneração dos calos (RC) e número de plantas por embrião (NPPE), para 96 genótipos de trigo. Os objetivos foram a procura de algum fator climatico específico em alguma característica da RCT e a determinação do período do desenvolvimento do trigo no qual estes fatores são mais eficazes. Os genótipos foram obtidos num campo experimental durante três estações climáticas. As relações entre as características da RTC e os fatores de clima dentro de três períodos de desenvolvimento do trigo (2, 6 e 10 semanas) antes da amostragem dos embriões foram analisadas pela técnica "biplot". As características das culturas de tecido foram influenciadas em diferentes graus pelos fatores climáticos: de 16.8% (RC) para 69.8% (FC). Ambientes da planta doadora com alta temperatura e pouca chuva reduziram ($p < 0.05$) o desempenho dos genótipos de trigo. A FC foi mais sensível aos fatores baseados em temperatura. As condições ambientais entre o florescimento e o estágio médio de leite dos frutos foram os mais importantes para FC, enquanto RC e NPPE não estavam relacionados a qualquer período.
Palavras chave: *Triticum aestivum* L., análise de correção, variável ambiental, period de crescimento

Introduction

A lot of attention in wheat (*Triticum aestivum* L.) improvement programs is given to a combination of biotechnological techniques and conventional methods. The successful application of many biotechnological techniques depends on callus formation and shoot regeneration capacity.

Although immature embryos are widely recognised as the most suitable explants to regenerate wheat plants (Bohorova et al., 1995; Kintzios et al., 1996; Li et al., 2003; Haliloglu et al., 2005), environmental conditions influencing the physiological status of field grown donor plants from which embryos were collected could be a limiting factor of their quality (Carman et al., 1988; Carman, 1995). Embryogenic competence of immature

wheat embryos is lower when donor plants are exposed to some kind of stress (biotic or abiotic). The donor plant growth in a drought season, as compared to favourable growing seasons, resulted in an increased variability and a decreased percentage of the callus formation and regenerating calli and the number of plants regenerated per embryo (Mitić et al., 2009). Dodig et al. (2008) reported all that significant associations between agronomic traits, considered as predictors of good *in vitro* plant regeneration, and tissue culture traits were responsive to the environment. There were several attempts to replace immature embryos with other explant sources (Özgen et al., 1998; Delporte et al., 2001; Sharma et al., 2005). However, calli derived from alternative explants displayed a lower regeneration potential than those obtained from immature embryos, which are still explants of choice for establishing high regenerative wheat tissue. In order to circumvent the limited availability influenced by a genotype and growing season restrictions, more frequent experiments on relationships between these factors are necessary.

The previously published analysis of the same set of 96 genotypes revealed that that the impact of donor plant growing conditions on tissue culture response (TCR) traits can be as large as the effect of the genotype (Mitić et al., 2009). In an attempt to increase knowledge in this subject, the objectives of this study were: (i) to evaluate which of the climatic environmental factors (rainfall, various temperature-based variables and sunshine duration) significantly influence tissue culture response of immature embryos of wheat and (ii) to determine the period of wheat growth during which these factors were the most effective.

Material and Methods

Plant materials: ninety-six wheat genotypes of worldwide origin, including cultivars, lines and landraces, were evaluated for their response to *in vitro* culture. These genotypes were chosen from the core collection of the Institute of Field and Vegetable Crops in Novi Sad, Serbia, on the basis of contrasting expression for one or other of 26 traits of agronomic importance such as earliness, plant height, tillering capacity, grain yield etc. (Kobiljski et al., 2002). In general, a high level of phenotypic variation was accumulated for CF, RC and PPE among analysed wheat genotypes (Mitić et al., 2009).

Field trials: plants were grown under rainfed conditions in the experimental field in Zaječar (144 m above sea level, 43°53'N, 22°17'E), Serbia, during the 2002/03, 2003/04 and 2004/05 seasons. Two-replicate trails were set up according to a randomised complete block design. Each plot consisted of three rows with the sowing density of 480 seeds per m^2. Rows were 1 m long and separated by 20 cm from each other. Standard agronomic practices were applied to provide adequate nutrition and to keep the plots free from diseases. The specific date of anthesis (anthers exerted from the spikelets) for each genotype was recorded when 50% of the ears were at this stage.

Tissue culture conditions: tissue culture was carried out from immature embryos isolated from immature seeds collected 13-15 days after anthesis from spikes on primary tillers and stored in the refrigerator at 4°C for 24 h. Immature seeds were disinfected by successive immersions into 70% ethanol for 1 min, 20% commercial NaOCl bleach solution (8% active chlorine) with a few drops of the fungicide Captan (30 min), and rinsed four times with sterile water. After disinfection, the immature embryos were removed aseptically from the seeds and placed with the scutellum side up on 20-mL solid nutrient medium in 9-cm Petri dishes (30 per dish in two replicates per genotype per year). The culture medium contained MS (Murashige and Skoog, 1962) mineral salts and vitamins, 100 mg L^{-1} casein hydrolysate, 30 g L^{-1} sucrose, 0.7% agar (Torlak, Serbia), and 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma, USA) for the callus initiation (two subcultures in three week-intervals). Induced calli were transferred to the MS growth regulator-free medium for another two subcultures. The pH of the media was adjusted to 5.8 prior to autoclaving at 115°C for 20 min. Cultures were grown at 25 ± 2°C, in white fluorescent light, with irradiance of 47 μmol m^{-2} s^{-1}, and a day/night regime of 16/8.

Tissue culture traits: the percentage of the callus formation (CF) was scored three weeks after the embryos were plated, the percentage of regenerating calli (RC) was calculated after six weeks of culture when the calli were transferred on a growth regulator-free medium, and the average number of plants per regenerating embryo (PPE) was measured after six weeks of culturing on the growth regulator-free medium and calculated as a mean number of plants per regenerating callus.

Environmental variables: data retrieved from the nearest meteorological station (distance up to 500 m) were used to derive the meteorological (temperature, rainfall and sunshine) variables for each of the years in which donor plants were grown. The following periods were used for each of the climatic variables: i) two weeks before the date of immature seed collecting (Zadoks scale from around 65 to 75, Zadoks et al., 1974); ii) six weeks before the date of immature seed collecting (Zadoks scale from around 35 to 75); iii) 10 weeks before the date of immature seed collecting (Zadoks scale from around 20 to 75). These periods were chosen to embody all stages of the wheat development from tillering to grain filling. Table 1 provides a description of the 15 variables assessed in this study. Table 2 provides the detailed data on tissue culture traits and environmental variables for each year.

Statistical analysis: to correct for non-normality, all statistical analyses were performed on *arcsin* transformed values of CF and RC. The analysis of variance (ANOVA) for a randomised complete block design with combined data from the three years was performed. The least significant difference (LSD) test

Table 1 – Description of the environmental variables and corresponding wheat growth stages (according to Zadoks scale).

Variable	Unit	Description	Growth stage
maxT2	°C	Mean maximum daily temperatures	65-75
maxT6	°C	Mean maximum daily temperatures	35-75
maxT10	°C	Mean maximum daily temperatures	20-75
minT2	°C	Mean minimum daily temperatures	65-75
minT6	°C	Mean minimum daily temperatures	45-75
minT10	°C	Mean minimum daily temperatures	15-75
varT2	°C	Mean daily temperatures variation (max.-min.)	65-75
varT6	°C	Mean daily temperatures variation (max.-min.)	45-75
varT10	°C	Mean daily temperatures variation (max.-min.)	15-75
SH2	Hours	Mean daily sunshine period	65-75
SH6	Hours	Mean daily sunshine period	45-75
SH10	Hours	Mean daily sunshine period	15-75
RF2	mm	Total precipitation	65-75
RF6	mm	Total precipitation	45-75
RF10	mm	Total precipitation	15-75

Table 2 – Classification of each of the three years for the tissue culture traits and environmental variables under examination (averaged over 96 genotypes).

Variable	Year		
	2003	2004	2005
CF	79.5 a	97.9 b	99.5 b
RC	17.5 a	38.0 b	36.3 b
PPE	3.1 a	4.7 b	6.5 c
maxT2†	28.0	27.0	23.1
maxT6	27.0	22.5	20.7
maxT10	23.1	20.7	21.1
minT2	13.7	11.1	10.6
minT6	11.4	8.9	9.5
minT10	8.3	7.8	7.8
varT2	14.3	13.4	13.7
varT6	15.6	13.5	13.6
varT10	14.8	13.0	13.3
SH2	9.0	7.6	8.4
SH6	8.9	6.5	7.5
SH10	7.6	6.1	6.5
RF2	23.6	41.6	28.8
RF6	66.3	81.6	85.9
RF10	152.3	129.9	142.2

CF = total of calli/total of embryos plated, RC = regenerating/total calli, PPE = plants/embryo. †See Table 1 for description of environmental variables. Values in the same row with same letter are not different ($p < 0.05$).

was used for comparing the means of tissue culture traits by years. A correlated structure of each dataset was assessed by the biplot analysis (Yan and Kang, 2003). Two-way tables of mean values of tissue culture traits and climate factors in each year were submitted to the following mathematical model: $(t_{ij} - \bar{t}_j)/s = \lambda_1 \xi_{i1} \eta_{j1} + \lambda_2 \xi_{i2} \eta_{j2} + \varepsilon_{ij}$, where t_{ij} the average value of trait i in year j, $\bar{t}_j$ is the average value of trait i over all years; s is the standard deviation of trait i; λ_1 and λ_2 are the singular values for the first and second principal components, PC1 and PC2, respectively; ξ_{i1} and ξ_{i2} are the PC1 and PC2 scores, respectively, for year j; η_{j1} and η_{j2} are the PC1 and PC2 scores respectively, for trait i; and ε_{ij} is the residual of the model associated with trait i in year j. The singular values of PC1 and PC2 are symmetrically distributed among the year and trait scores (Yan, 2002). The biplot is constructed by plotting PC1 against PC2 scores for years and traits. In addition, vectors were drawn from the origin of the biplot to each year and trait to visualise relationships among them. The coefficient of correlation between any two traits and/or years is approximated by the cosine of the angle between their vectors (e.g., r = cos180° = –1, cos0° = 1, and cos90° = 0). The same was done for year × factor tables in each particular year.

Results and Discussion

The main effects of genotype, year and their interaction were all significant at the $p < 0.01$ for CF, RC and PPE. The relative magnitudes of the sources of variation, however, vary greatly, as indicated by the variance components as a percentage of the total variation. The main effect of year explained 69.8% of the total sum of squares for CF while a genotype main effect dominated

the variation for RC (62.7%) and PPE (39.7%). Nevertheless, a non-genotype source of variation, year and genotype × year, still account for a relatively large portion of total variance for RC (16.8 and 20.5%, respectively) and particularly for PPE (25.3 and 35%, respectively).

A trait × environment biplot based on mean values in each of the years was used to visualise similarities/dissimilarities among tissue culture traits in response to the environment (Figure 1). All tissue culture traits were negatively influenced by environmental conditions in the first year (2003), as evidenced by the obtuse angles between their vectors (the rays connecting the traits to the biplot origin) and the vector of this year. This year was characterized by a higher value of the temperature based parameters than the other two years for the all three periods (Table 2). Rainfall was lower in 2003 compared to 2004 and 2005 for two-week (from flowering to the medium milk stage) and six-week (from stem elongation to the medium milk stage) periods prior to sampling of embryos. Hence, warm temperatures and lower water availability in 2003 reduced ($p < 0.05$) tissue culture performance of wheat genotypes compared to more favourable 2004 and 2005 years (Table 2). This is in agreement with Hess and Carman (1998) that embryogenic competence of immature wheat embryos is lower when donor plants are exposed to heat or water stress. Interestingly, among the 96 genotypes only cultivar Florida (USA) displayed higher ($p < 0.05$) values of RC and PPE in 2003 compared to 2004 and 2005 (Mitić et al., 2009). The authors hypothesised that environmental stresses increased the concentration of ABA in plants of this genotype, and this induced a better adaptation to the tissue culture and improved the regeneration response. A similar explanation for the observation that donor plants of durum wheat, when subjected to a mild heat or drought stress, displayed better responses to the biolistic transformation procedures was reported by Pellegrineschi et al. (2002). RC performed particularly well in 2004 and PPE in 2005, as evidenced by the acute angles between their vectors. CF had a similar positive response to 2004 and 2005. Years assumed both values, positive and negative, for both axes, implying that the trait performance varied dramatically among these years. So, further analysis on climatic factors for each year individually was performed.

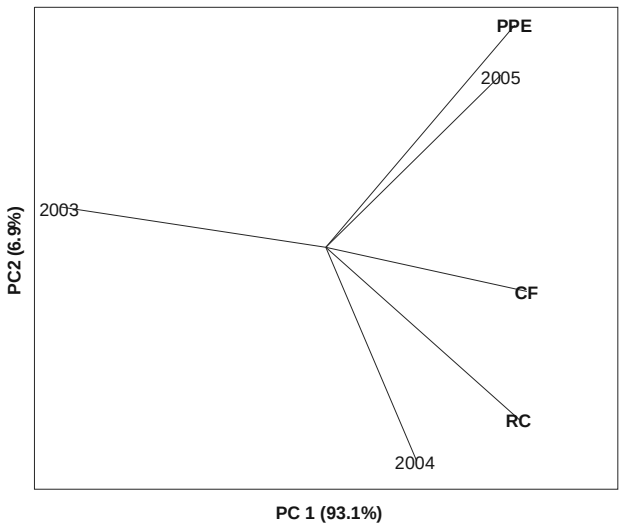

Figure 1 – Trait × environment biplot based on mean values in each of the year . CF = total of calli/total of embryos plated, RC = regenerating/total calii, PPE = plants/embryo.

To relate the climatic variables to the tissue culture traits in each year, environment × factor biplots based on data in Table 2 are presented in Figure 2. The biplots were based on the first two principal components derived from subjecting the standardised environmental factors-by-trait table. In the biplots, a vector is drawn from the biplot origin to each marker of the factors to facilitate visualisation of the relationships between and among the tissue culture traits and climatic variables. The biplot for each of the three years explained 69 to 78% of the total variation of the two-way table.

Each year was characterised by strong positive associations between PPE and RC. The regeneration capacity and CF were negatively related in 2003 and 2005, while there was no obvious association in 2004. The environment × factor biplot for 2003 (Figure 2a) shows that the 15 climatic factors fell into three relatively independent groups: SH2, SH6, SH10, maxT2, maxT6, maxT10, minT2, minT6, minT10, and varT2 constitute one group with positive associations among them. The second group of positively associated traits consists of RF6, RF10 and varT10. This group had high and positive correlations with RC and PPE. In the third group, varT6 and RF2 can be assorted, as these factors only showed positive correlations with CF. All other climatic factors, except varT10 and SH6, had a more or less negative influence on CF. On the other hand, RC and PPE were negatively related only to varT6, SH6 and maxT6. It seems that CF was more sensitive to warm and dry environmental conditions than RC and PPE. In such a season, any rainfall starting from early stages of donor plants vegetative growth (from tillering onward) will improve RC and PPE. On the other hand the highest contribution of rainfall to the CF performance was in period 1, from flowering to the medium milk stage, when air temperatures were the highest (Table 2). Drought and heat stress may influence competence by modifying endogenous hormone and energy levels in explants and in the tissues that give rise to them (Carman, 1995). Jiménez and Bangerth (2001) reported that embryogenic callus had higher concentrations of endogenous free indoleacetic acid (IAA), in comparison to the concentrations found in nonembryogenic callus.

The environment × factor biplot for 2004 (Figure 2b) also revealed three distinct (non-overlapping clusters) groups of the 15 climatic factors. One group consists of SH6, SH10, maxT2, maxT6, maxT10, minT2, minT6 and minT10 with positive associations among them and nega-

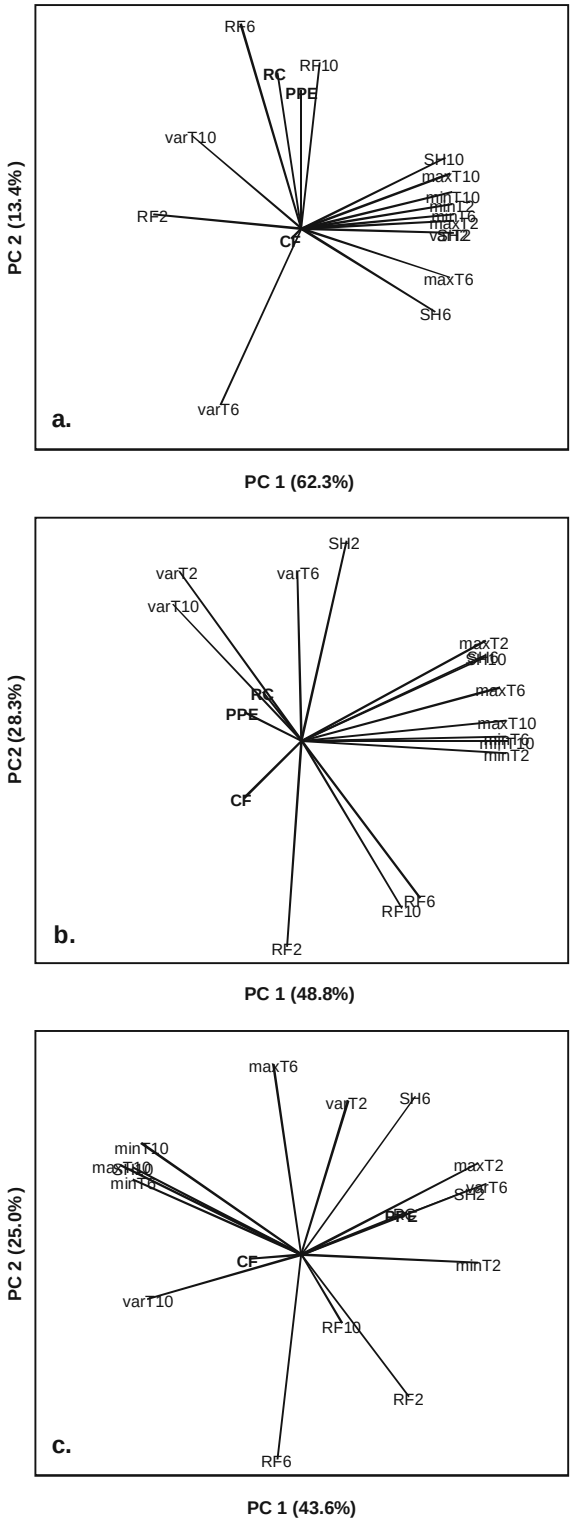

Figure 2 – Vector view of an environment × factor biplot summarising the interrelationship among plant tissue traits and climatic factors presented in Table 2. (a) 2003; (b) 2004; (c) 2005. PC1 and PC2 are the first and the second principal components, respectively. CF = total of calli/total of embryos plated, RC = regenerating/total calii, PPE = plants/embryo. See Table 1 for description of environmental variables.

tive relationships with all tissue culture traits. This group was more or less independent of other two groups. Mean daily temperature variations in all periods and SH2 make up another group of positively associated traits. This group had a positive impact on RC and PPE. The third group consists of RF2, RF6 and RF10 with positive associations among them and negative relationships with the second group of traits. In 2004, all tissue culture traits were negatively influenced by minimum and maximum air temperatures in all periods, as well as with SH6 and SH10. Rainfall, particularly during two weeks before the date of collecting immature seeds (RF2), had a positive impact on CF. RC and PPE were positively associated with daily temperature variation in all periods. Interestingly, in contrast to the previous year, RC and PPE were negatively related to rainfall in all periods. This is probably because the higher rainfall in 2004 caused lower temperatures and a shorter sunshine period (Table 2). A particularly negative correlation between rainfall and sunshine duration was observed from flowering to the medium milk stage (Figure 2b). For barley, the regeneration from embryo-derived callus of greenhouse-grown donor plants was more dependent on the average solar radiation than on temperature (Dahleen, 1999).

In 2005, there was no obvious clustering of climatic factors on the biplot (Figure 2c). In contrast to the previous two years, rainfall had neither positive nor negative effects on tissue culture traits. Also, minimum and maximum temperatures showed positive effects, while in previous two years their impacts on CF, RC and PPE were mainly negative. The reason for this may be because 2005 was the coolest year, particularly in the period from flowering to the medium milk stage (Table 2). It may be possible to increase competence of immature wheat embryos for plant regeneration by growing donor plants under cool conditions, which appears to delay the accumulation of endogenous hormone levels in kernels (Hess and Carman, 1998). The callus formation was positively influenced by varT10, minT6, maxT10, minT10, as well as SH10. The regeneration capacity also had a positive correlation with the temperature based factors (varT6, maxT2, minT2, varT2) and the sunshine duration (SH2 and SH6). For barley, a sufficient regeneration from greenhouse-grown donor plants was obtained when temperatures were moderate and natural light levels were high (Dahleen, 1999). Factors positively correlated to CF and regenerative potential were mainly effective during ten and two weeks prior to sampling of embryos, respectively.

In general, there was no consistency in associations between the climatic factors and/or periods with tissue culture performance for wheat. These relationships varied over years. For example, if donor plants were grown under warm and dry conditions, rainfall would contribute positively to both CF (two weeks prior to sampling of embryos) and regenerative potential (six and ten weeks prior to sampling of embryos). In cooler and wet-

ter seasons rainfall was mainly neutral or even negatively correlated to tissue culture traits. Nevertheless, in each year, maxT2, minT2 and SH2 were negatively correlated with CF. This implies in the fact that CF was highly sensitive to high temperatures between flowering and the medium milk stage. On the other hand, maxT2 and minT2 had positive effects on RC and PPE in the coolest year (2005) but neutral or slightly negative effects in the other two years. Moderate temperatures during two weeks prior to sampling appeared to be highly effective for the callus induction and the regeneration potential of immature embryos of wheat.

The environmental conditions during the donor plant growth may play an important role in the tissue culture response of wheat. Donor plants grown under a warm and dry environment resulted in a significantly reduced callus induction and regeneration potential. Components of variance and the correlation analysis between tissue culture traits and climatic factors revealed that CF was more influenced by climatic factors than RC and PPE. Rainfall effects on tissue culture traits depends on the season and varies from positive in dry and warm conditions to negative in cooler seasons. The callus induction rate was more sensitive to temperature based factors than the regeneration capacity. It seems that weather conditions between flowering and the medium milk stage were the most important for CF, while RC and PPE were not particularly related to climatic factors in any period. This conclusion needs to be tested under more environment types in the future.

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