BioTechnologia vol. 93(2) • pp. 116-122 • 2012
Journal of Biotechnology, Computational Biology and Bionanotechnology RESEARCH PAPER

Examination of ability to androgenesis of spring wheat genotypes resistant to *Fusarium*

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Abstract

Diseases caused by fungi from the genus *Fusarium* constitute a serious problem in spring wheat cultivation. Ear infestation leads reduced yield and plant contamination with mycotoxins. Therefore it is essential to introduce resistance genes to high-yielding cultivars. The generation of double haploid (DH) lines makes it possible to shorten the time required to select favourable genotypes. The aim of this study was to analyze the capacity of plants to regenerate in anther cultures and to generate DH of spring wheat genotypes that potentially constitute germplasm material for resistance breeding, directed against fungi from the genus *Fusarium*. The plant material comprised wheat cultivars with increased resistance to *Fusarium*: Sumai 3, Ning 8331, Norin 52, Frontana, as well as line 8475-59 and high-yielding Polish cultivars: Łagwa, Waluta and Zadra. Spikes were subjected to a thermal shock at 4°C. Anthers were placed on the C17 inducing medium. Two combinations of growth regulators, i.e. 2,4-D and kinetin as well as 2,4-D and dicamba were applied. A total of 19,200 anthers were used, resulting in a total of 440 calli from which 352 plants were obtained. Of this number 14% were albino. The regeneration efficiency ranged from 0% to 17.33% depending on the analyzed genotype and the combination of growth regulators in the induction medium. The highest number of green plants and seeds were obtained from cv. Łagwa, Zadra and Sumai 3. Analyses of the regenerants, using a flow cytometer, demonstrated that depending on the genotype, haploids constituted approx 75% of green plants. Doubling of the number of chromosomes at a 40% efficiency rate took place when 0.1% colchicine was used. In the experiment 54 DH lines were obtained from which 283 kernels were collected. The best genotypes for crossing components in terms of their regeneration ability were found to be cultivar Frontana among the cultivars resistant to *Fusarium*, and cultivar Łagwa from Polish cultivars used in the experiment.

Key words: spring wheat, androgenesis, regeneration efficiency, double haploids

Introduction

Bread wheat is the most commonly grown cereal worldwide and in Poland. Infestation with fungal pathogens from the genus *Fusarium* leads to reduced yield through the reduction of grain size and the number of kernels in the ear as well as contamination of the plant with mycotoxins that are harmful to human and animal health (Klahr et al., 2007). The European Union Member States are obliged to follow standards concerning toxin content in the grain and its processed products. Most wheat cultivars grown in Europe exhibit high or very high susceptibility to fusariosis (Stępień and Chełkowski, 2005). In Poland, grain contamination with *Fusarium* mycotoxins is a serious problem and has been frequently reported (Chełkowski et al., 1988). This indicates the need to improve the resistance of wheat cultivars to ear blight caused by *Fusarium* sp. The sources that may provide that resistance includes spring wheats from China (Sumai 3 and Ning lines), and Brazil, cv. Frontana (Masterhazy, 2002; Wiśniewska and Kowalczyk, 2005). Unfortunately, all these genotypes are characterized by unfavorable yields and poor flour quality (Wiśniewska and Kowalczyk, 2005). The transfer of resistance genes from those cultivars to high-yielding cultivars is connected with crossing and selection of resistant genotypes extended over periods of many years. The application of double haploid (DH) lines makes it possible to shorten the breeding program and facilitates faster selection of desirable genotypes. Unfortunately, producing haploid wheat plants through androgenesis is strongly dependent on the genotype and is not very efficient (Rybczyński et al., 1991; Zamani et al., 2003; Konieczny et al., 2003). Moreover, there are very few reports on the capacity of androgenesis in genotypes that

are sources of resistance to *Fusarium* (Masojć et al., 1993). Therefore, the aim of this experiment was to verify the capacity of plant regeneration in anther cultures of spring wheat genotypes as sources of resistance to *Fusarium*, as well as crossing them with Polish cultivars.

Materials and methods

Plant material comprised wheat cultivars that are known sources of resistance to fungal infection caused by fungi from the genus *Fusarium*: Chinese varieties Sumai 3 and Ning 8331, Japanese cultivar Norin 52, Brazilian cultivar Frontana and line 8475-59 as well as high-yielding Polish cultivars: Łagwa, Waluta and Zadra.

Donor plants were grown in the greenhouse in pots. Spikes were excised when microspores were in the medium or late uninuclear stage (Barnabas et al., 2001). The development stage of microspores was determined using smear preparations stained with Belling's fluid. Androgenesis was initiated by subjecting the spikes to thermal stress at 4°C for a period of 7 or 14 days. The spikes were sterilized for 4 min in 4.85% NaClO. Subsequently, the spikes were rinsed in sterile distilled water. Anthers without filaments were transferred onto the C17-inducing medium (Wang and Chen, 1983). Two combinations of growth regulators were applied: 2,4-Dichlorophenoxyacetic acid (2,4-D) (1.5 mg/l) with kinetin (0.5 mg/l), and 2,4-D (1.0 mg/l) with dicamba (1.0 mg/l). In each combination the explants were placed on 10 Petri dishes with 60 anthers, collected from one ear, per dish. A total of 19,200 anthers were used (2,400 from each analyzed genotype). The source of carbohydrates was maltose (90 g/l). The medium was solidified with gerlite at 2.5 g/l. The explants were incubated at 3°C in the dark.

After 8 weeks the calli were passaged on the MS regeneration medium (Murashige and Skoog, 1962) with an addition of 0.5 mg/l 1-naphthaleneacetic (NAA) and 0.5 mg/l kinetin. The dishes were placed in the culture chamber at 24°C with a photoperiod of 16-h light/8-h darkness. After 3-6 weeks, plant regeneration was observed. The regenerants were subsequently passaged to glass flasks to facilitate further growth.

The ploidy level of the obtained plants was analyzed by measuring the nuclear DNA content by laser flow cytometry. For the analysis, young leaves of regenerated plants and of internal standard were chopped simultaneously with a sharp razor blade in a plastic Petri dish with 0.5 ml nucleus-isolation buffer supplemented with 50 µg/ml ribonuclease (Śliwińska, 2008). The 2C genome of cv. Muszelka was used as an internal standard. After chopping, the suspension was passed through a 30-50 µm mesh nylon filter and incubated for approximately 30-minutes. Following the incubation, a staining buffer (propidium iodide; 50 µg/ml) was added and measurements were taken using a Partec flow cytometer (Münster, Germany) equipped with an argon laser (Śliwińska, 2008). Ploidy measurements were conducted at the Department of Molecular Biology and Cytometry, the University of Technology and Life Sciences in Bydgoszcz, Poland.

Selected haploids were placed for 6 h in a 0.1% colchicine solution with an addition of DMSO and Tween. Rinsed plants were planted in sterile soil. The plants that set seeds were considered as DH.

The efficiency of callus formation was assessed and found to constitute the average number of calli per 100 placed anthers. The plant regeneration efficiency was calculated as the average number of plants produced per 100 used anthers. Both parameters were expressed as a percentage.

Results

From all the anthers used, a total of 440 tubercular, cream-colored calli were obtained. Among the genotypes resistant to *Fusarium*, explants of cv. Ning 8331 formed the greatest number of calli (67) at a regeneration rate of 2.79%. The smallest number of calli was observed for the anthers of cv. Norin 52 – the efficiency of callus formation for this cultivar was 0.25%. Polish cultivars Łagwa, Waluta and Zadra formed a higher number of calli (106, 61, 90, respectively) and the efficiency of this process ranged from 2.54% to 4.42%. The efficiency of callus formation in Polish cultivars was on an average over 3-fold higher than that of genotypes resistant to *Fusarium* (Table 1).

Analysis of the effect of growth regulators on callus formation in the tested genotypes revealed that the strongest stimulation of this process was provided by the C17 medium with 2,4-D (1.0 mg/l) and dicamba (1.0 mg/l) following a 7-day thermal stress. In this combination calli were formed most efficiently by Polish cultivars: Waluta and Łagwa, in which the efficiency of callus

Table 1. Total average efficiency of callus formation of the analyzed spring wheat cultivars

Cultivar	Number of used anthers	Number of calli	Average efficiency of callus formation [%]
Sumai 3	2400	48	2.00
Ning 8331	2400	67	2.79
Norin 52	2400	6	0.25
Frontana	2400	48	2.00
8475-59	2400	14	0.58
Łagwa	2400	106	4.42
Waluta	2400	61	2.54
Zadra	2400	90	3.75
Total	19200	440	2.29

Table 2. The effect of growth regulators and spikes pretreatment on the efficiency of callus formation and green plant regeneration in the analyzed spring wheat genotypes

Cultivar	Average efficiency of callus formation [%]				Average efficiency of green plant regeneration [%]			
	spikes pretreatment 7 days at 4°C		spikes pretreatment 14 days at 4°C		spikes pretreatment 7 days at 4°C		spikes pretreatment 14 days at 4°C	
	C17 medium + 2,4-D (1.5 mg/l) + kinetin (0.5 mg/l)	C17 medium + 2,4-D (1.0 mg/l) + dicamba (1.0 mg/l)	C17 medium + 2,4-D (1.5 mg/l) + kinetin (0.5 mg/l)	C17 medium + 2,4-D (1.0 mg/l) + dicamba (1.0 mg/l)	C17 medium + 2,4-D (1.5 mg/l) + kinetin (0.5 mg/l)	C17 medium + 2,4-D (1.0 mg/l) + dicamba (1.0 mg/l)	C17 medium + 2,4-D (1.5 mg/l) + kinetin (0.5 mg/l)	C17 medium + 2,4-D (1.0 mg/l) + dicamba (1.0 mg/l)
Sumai 3	1.00	4.67	0.83	1.50	0.00	6.67	0.00	0.00
Ning 8331	1.00	6.33	0.83	3.00	0.00	0.00	0.00	0.00
Norin 52	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Frontana	4.33	0.50	0.17	3.00	17.33	0.00	0.00	0.00
8475-59	0.83	0.00	0.67	0.83	0.00	0.00	0.00	0.50
Łagwa	0.50	8.50	0.83	7.83	0.33	13.67	0.00	8.33
Waluta	0.00	10.00	0.17	0.00	0.00	2.50	0.00	0.00
Zadra	7.50	0.67	1.83	5.00	0.50	0.00	0.00	1.33

tissue formation was 10.0% and 8.5%, respectively. Among cultivars which were sources of resistance to *Fusarium* in this combination, calli were formed most efficiently by plants of cv. Ning 8331 and Sumai 3, at an efficiency rate of 6.33% and 4.67%, respectively. In turn, plants from Polish cv. Zadra and Brazilian cv. Frontana formed the highest number of calli on the C17 medium with an addition of 2,4-D (1.5 mg/l) and kinetin (0.5 mg/l) after 7 days of ear pretreatment. The number of calli per 100 used anthers was 7.5 and 4.33, respectively. The lowest number of calli was observed on the medium containing 2,4-D and kinetin after a 14-day thermal stress, on which callus formation occurred at a rate ranging from 0% on anthers of *Fusarium*-resistant cv. Norin 52 to 1.83% on explants from Polish cv. Zadra (Table 2).

In this study, from a total of 440 calli, 352 plants were regenerated, of which 14% presented chlorophyll defects. The greatest number of green plants was regenerated from calli of Polish cv. Łagwa and Brazilian cv.

Frontana as sources of resistance, at 134 and 104 plants, respectively. Regeneration was not observed on the explants of cv. Norin 52, while from calli of cv. Ning 8331 only albino plants were obtained. The regeneration efficiency of green plants from Polish cultivars was greater than that of the genotypes resistant to *Fusarium* (Table 3).

Among genotypes that are sources of *Fusarium* resistance, the greatest number of green plants regenerated from the calli of cv. Frontana, but in this case regeneration occurred only on the C17 medium with an addition of 2,4-D (1.5 mg/l) and kinetin (0.5 mg/l) following a 7-day thermal stress. The efficiency of regeneration was 17.3%. Among genotypes resistant to *Fusarium,* high regeneration rates of green plants (6.67%) was also observed for calli of cv. Sumai 3 on the C17 medium with an addition of 2,4-D and dicamba after 7 days of ear exposure to low temperature. Polish cultivars Łagwa and Waluta formed the highest number of green plants in the same combination of growth regulators and thermal stress duration. The efficiency of regenerant formation was 13.67% and 2.5%, respectively. In turn, the regeneration of plants from cv. Zadra and line 8475-59 was most efficient after a 14-day ear treatment at low temperature, on a medium with an addition of dicamba (regeneration rate of 8.33% and 0.5%, respectively). The C17 medium, with an addition of 2,4-D and kinetin after a 14-day ear exposure to low temperature did not stimulate regeneration in any of the tested cultivars (Table 2).

The ploidy level was determined for a total of 210 plants. The other regenerants were rejected because after transfer to the soil, their development was inhibited or they displayed accelerated earing. A considerable number of plants subjected to the measurement of DNA content in the nuclei were haploids (75.71%). It was found that 46 plants were diploid and 5 tetraploid (Table 4). The highest number of haploids was obtained from cv. Łagwa (86) and Frontana (52). All haploids were subjected to colchicine treatment and in this way 54 plants with a doubled number of chromosomes were obtained. Thirty plants were generated from the Polish cv. Łagwa, 23 from cv. Frontana carrying *Fusarium* resistance genes, while one plant developed from line 8475-59. Thus the effectiveness of colchicine treatment was 40%. All the obtained DH lines set seeds. The number of kernels in an ear ranged from 1 to 19. Among the total of 283 kernels, 174 were produced from cv. Łagwa, 101 from cv. Frontana and 8 from line 8475-59.

Discussion

Plant genotype is a major factor influencing the capacity of androgenesis (Lazar et al., 1984; Tuvesson et al., 1989). In the conducted study these observations were definitely confirmed. Eight tested spring wheat genotypes differed considerably, both in terms of their ability to form calli and the efficiency of regeneration of green and albino plants. Polish cultivars formed, on an average, 3 times more calli than *Fusarium*-resistant genotypes. Similar to many other studies, the average efficiency of callus tissue formation was low, ranging from 0.25% for *Fusarium*-resistant cv. Norin 52 to 4.42% for the Polish cv. Łagwa.

A similarly low efficiency has been reported by Konieczny et al. (2003) who obtained from 0.19 calli per 100 used anthers of Polish cv. Wanda to 9.07 of cv. Apollo. The varied capacity for callus tissue formation in wheat, from 2.66 to 14.91 structures per 100 used anthers, has also been observed by Zamani et al. (2003). In turn, in the experiments conducted by Ponitka and Ślusarkiewicz-Jarzina (2009) in oat anther cultures, the callus tissue was formed with an efficiency ranging from 0 to 12.1 structures per 100 anthers.

Even greater genotypic differences were found in the efficiency of regeneration of green plants. No such plants were regenerated from the two *Fusarium*-resistant genotypes, i.e. Ning 8331 and Norin 52. Failure of plant regeneration from cv. Ning 8331 in the experiment conducted by Masojć et al. (1993) confirms poor capacity for androgenesis in the case of this cultivar. Masojć et al. (1993) did not obtain plants from cv. Sumai 3, but in the studies conducted using the C17 inducing medium, the average efficiency of regeneration of green plants in this cultivar was 1.67%, while in the variant with an addition of 2,4-D and dicamba after 7 days of spike pretreatment it was 6.5%. Thus it is very likely that *Fusarium*-resistant cultivars Norin 52 and Ning 8331, from which no plants were obtained, require other inducing media. The lack of response of wheat genotypes to the induction of androgenesis has been reported by many authors. Tuvesson et al. (2000) tested 257 winter wheat lines, of which 186 formed green plants at the efficiency of regeneration ranging from 0% to 34.4%.

Table 3. Total average efficiency of plant regeneration of the analyzed spring wheat cultivars

Cultivar	Number of used anthers	Number of albino plants	Average efficiency of albino plant regeneration [%]	Number of green plants	Average efficiency of green plant regeneration [%]
Sumai 3	2400	1	0.04	40	1.67
Ning 8331	2400	18	0.75	0	0.00
Norin 52	2400	0	0.00	0	0.00
Frontana	2400	3	0.13	104	4.33
8475-59	2400	1	0.04	3	0.13
Łagwa	2400	12	0.50	134	5.58
Waluta	2400	6	0.25	15	0.63
Zadra	2400	7	0.29	8	0.33
Total	19200	48	0.25	304	1.58

Table 4. Ploidy level of regenerants of the analyzed spring wheat cultivars

Cultivar	Number of green plants	Number of analyzed plants	Haploids	Diploids	Tetraploids
Sumai 3	40	17	15	2	0
Ning 8331	0	0	0	0	0
Norin 52	0	0	0	0	0
Frontana	104	73	52	19	2
8475-59	3	3	3	0	0
Łagwa	134	103	86	17	0
Waluta	15	6	2	1	3
Zadra	8	8	1	7	0
Total	304	210	159	46	5

In turn, Adamski et al. (2009) did not obtain plants from 5 out of 33 tested genotypes, Chaudhary et al. (2003) obtained green plants from 6 genotypes from 11 tested cultivars, while Ljevanaić et al. (2006) observed regeneration in 7 out of 8 tested genotypes. The efficiency of green plant regeneration from anthers was low, with an average for all the tested genotypes 1.58%. Similar regeneration efficiency at 1.8% was observed by Adamski et al. (2009). In the present study, variations of the regeneration efficiency between genotypes were evident, ranging from 0% up to 5.58% on an average, similar, to the results of studies conducted by other authors (Adamski et al., 2009; Chaudhary et al., 2003; Masojć et al., 1993). The efficiency of plant regeneration from Polish cultivars was greater than from *Fusarium*-resistant genotypes.

The induction of changes in microspore development requires a stress stimulus. For this purpose, pretreatment is applied to provide appropriate physiological conditions for the microspore (Śnieżko, 1991). In this experiment, spikes were subjected to a 7 and 14 day thermal stress at 4°C. Both, callus formation and regeneration of green plants, occurred more efficiently on the application of a shorter cold temperature treatment. Only the calli obtained from line 8475-59 and cv. Zadra formed more green plants after 14 days of thermal stress. This result confirmed earlier observations of many authors that androgenesis ability and efficiency of plant regeneration depends mainly on the plant genotype. Kim et al. (2003) recommended shorter stress duration – from 1 to 5 days for two spring wheat cultivars (Bobwhite and Pavon 76). The same period of time

was recommended Chaudhary et al. (2003) who tested nine lines of winter wheat, two of spring wheat and 18 F_1 hybrids. In turn, Redha and Talaat (2008) proposed a longer exposure to low temperature for the spikes – from 7 to 10 days. They investigated four hexaploid spring wheat genotypes. In contrast, Pauk et al. (2003) suggested that a thermal shock for wheat should last as long as 14 days. Datta (2005) tested factors controlling crop development and recommended, for wheat, increasing the temperature to 8°C for approx. 10 to 14 days.

For the androgenesis induction, the presence of exogenous auxin is required, which may be applied together with cytokinines inducing cell division. In the present study, 2,4-D was added to both tested media at 1.0 mg/l or 1.5 mg/l in order to initiate the process of androgenesis. Regeneration was observed only through indirect embryogenesis. Zheng and Konzak (1999) observed callus induction with no involvement of 2,4-D. However, the number of formed structures was low and they did not display the capacity for plant regeneration. The same authors also stated that 1-2 mg/l was the most appropriate concentration of 2,4-D in the inducing medium. A too high concentration (4 mg/l) resulted in the loss of callus formation or plant regeneration.

In order to improve the androgenesis efficiency, the induction medium was supplemented with 0.5 mg/l kinetin or 1.0 mg/l dicamba, added to the already present 2,4-D. Among the 8 tested genotypes, 4 formed more calli on the medium with dicamba and 4 with kinetin, while in 5 out of the 6 regenerating genotypes, regeneration of green plants was more efficient on a medium with an addition of dicamba. Satyavathi et al. (2004), when investigating the effect of 2,4-D, dicamba and picloram on callus induction and plant regeneration in blastodisc cultures of durum wheat, recorded the highest callus induction and plant regeneration on a medium containing 2 mg/l dicamba. In turn, Immonen and Anttila (1998) replaced 2,4-D dicamba in the combination with kinetin and observed that the percentage of the formed calli, embryos and green plants by anthers was the same as in case of 2,4-D application. Hassawi et al. (1999) investigated the effect of three different auxins (2 mg/l 2,4-D, 2 mg/l dicamba or 2 mg/l picloram) and 1 mg/l kinetin on the response of wheat and triticale in anther cultures. The highest percentage of responding anthers was observed on the medium with 2,4-D and kinetin (13.9%), followed by the medium with picloram and kinetin (11.5%), while it was the lowest for the combination of 2,4-D and dicamba (10.1%). This study does not confirm these observations, since a higher efficiency of callus formation was recorded on a medium with 2,4-D and dicamba (depending on the genotype, ranged from 0% to 10%). In comparison, the efficiency of callus induction on the medium with 2,4-D and kinetin ranged from 0% to 7.5%. A positive effect of kinetin, in combination with 2,4-D, was evident for the anthers of cv. Frontana which regenerated into green plants most efficiently (17.33%). Rakoczy-Trojanowska et al. (1996), while investigating a different combination of inducing media: 2 mg/l 2,4-D, 0.5 mg/l kinetin and 3 mg/l picloram, also observed the highest plant regeneration efficiency on a medium containing 2,4-D and kinetin (a total of 33 plants, including 24 albino). In this study we observed a varied response of genotypes to the composition and concentration of growth regulators in the induction medium.

Conclusions

1) All the studied Polish wheat cultivars demonstrated the ability to form calli and to generate green plants. Genotypes resistant to *Fusarium* showed a lower capacity of androgenesis in comparison to the Polish cultivars.
2) As suspected, investigated cultivars displayed a diverse reaction to the applied combinations of growth regulators. On an average the regeneration of calli and green plants was found most effective on the C17 medium with an addition of 1.0 mg/l 2,4-D and 1.0 mg/l dicamba after 7 days of ear pretreatment at temperatures lowered to 4°C. In turn, cv. Frontana regenerated green plants only on the C17 medium with an addition of 1.5 mg/l 2,4-D and 0.5 mg/l kinetin.
3) Cultivar Frontana, among cultivars resistant to *Fusarium,* and cultivar Łagwa from Polish cultivars proved to be the best genotypes for crossing components, in terms of their regeneration ability.

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