Combined effect of 2,4-D and sucrose concentration on the gynogenetic response in durum wheat

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Abstract

In order to produce double haploid plants from unfertilised ovary cultures of durum wheat (Triticum turgidum ssp. durum), we studied the effect of two culture media: MS and C17 on nine cultivars. For both culture media, the effect of three concentrations of 2,4-D (2,4-dichlorophenoxyacetic acid): 2, 3.5 and 5 mg L-1, and four concentrations of sucrose : 45, 60, 90 and 120g L^{-1} were analyzed.

The responses of unfertilised ovary culture were dependent on the cultivar. However, other factors such as medium, sucrose and 2,4-D concentrations affected the cultivars's gynogenetic abilities. From the nine cultivars studied, 67 green plants were regenerated; only one cultivar ('Acsad 65') did not produce response.

Key words : *Triticum durum*, gynogenesis, unfertilized ovaries, double haploids, green plant.

ملـخص

من اجل الحصول على نباتات القمح الصلب ذات النصف العدد اصبغي انطلاقا من المبيض غير الملقح، درسنا فعالية نوعين من الأوساط المحيطية C 17 و S M، و تسعة أصناف من القمح الصلب. بالنسبة لكل وسط، حللنا اثر أربع تركيزات من السكروز و ثلاثة تركيزات من 2,4 ديكلوروفينوكسي اسيتيك اسيد . أظهرت النتائج على أن زراعة المبيض يتوقف على نوع الفصيلة، بالإضافة أننا لاحظن وجود تأثيرات أخرى لاسيما الوسط المحيطي المستعمل تركيز السكروز و تركيز(2,4 -D).

انطلاقا من هذه التجربة، حصلنا على 67 نبتة خضراء و من بين التسعة أصناف المدروسة تبين أن أكساد صنف يجيب سلبي.

الكلمات المفتاحية: قمح الصلب، مبيض غير ملقح، نصف عدد صبغي، نبتة خضراء.

Résumé

Dans le cadre de la production de plantes haploïdes doublées du blé dur (Triticum turgidum ssp. durum) par culture in vitro d'ovaires non fécondés, nous avons étudié pour neuf génotypes l'effet de deux milieux de culture, MS et C17. Aussi a-t-on analysé, pour ces deux milieux, l'action de trois concentrations de 2,4-D (acide 2,4 dichlorophénoxyacétique) : 2 , 3.5 et 5 mg.L^{-1}, en combinaison avec quatre concentrations en saccharose : 45 , 60 , 90 et 120 g.L^{-1}.

Parmi les différents facteurs étudiés, l'effet du génotype est très déterminant, cependant divers autres facteurs (la nature du milieu de base, les quantités de saccharose et du 2,4-D) agissent en interaction avec le génotype sur la capacité gynogénétique. A partir des neufs génotypes étudiés, 67 plantes toutes chlorophylliennes sont régénérées et seul le cultivar 'Acsad' s'est avéré récalcitrant.

Mots clés : *Triticum durum*, gynogenèse, ovaires non fécondés, haploïdes doublés, plantes chlorophylliennes.

Introduction

Production of haploid plants through in vitro culture of anthers or ovaries which respectively enclose male and female gametophytes is potentially very useful in breeding and selection programs in general and for cereals in particular (Piri et al. 1994, Aljar et al., 1995, Bidhan and Mandal 2004). Once obtained, haploids can undergo chromosome doubling and become fertile homozygous plants. The use of such plants facilitates selection and can reduce by several years the creation of new varieties (Sibi and Demarly 1997, Chlyah et al., 2001, Melinda et al., 1992).

In cereals, double haploid have generally been obtained through anther and microspore culture (Wang et al., 1993, Mioeni and Sarrafi 1995, Castillo et al., 2000) or by intergeneric crosses (Djmadi et al., 2004). However, this method is inefficient in some species such as durum wheat. Poor results through androgenesis are due sometimes to low embryo yields (Ghaemi and Sarrafi 1993a, b) but mainly to non-chlorophyllous plant formation (Albino) (Saidi et al., 1997).

Recourse to the culture of unfertilised ovaries or ovules (gynogenesis) is an alternative for haploid production in species with low or no capacity for androgenesis (Joachim and Larissa 1998) or in the case of male sterility when no microspores are formed. Gynogenesis is influenced by several factors: cultivars, physiological stage of ovules, culture medium composition and culture conditions (Serik and Mukhambetzanov 1997, Mukhambetzanov and Erezhepov, 1992, Sibi et al. 2001); in a prior article on durum wheat, Mdarhri-Alaoui et al., (1998) reported the favourable action of a cold pre-treatment, of a particular light regime, and a clear genotypic effect.

In the present article, the correlated actions of 2,4-D and sucrose concentrations were studied in two culture media for nine cultivars of durum wheat.

Materials and methods.

Nine cultivars of durum wheat *(Triticum turgidum ssp. durum)* ('Anouar', 'Jawhar', 'Yassmine', 'Belbachir' 'Acsad65', 'Sebou' 'Jori', 'Kyperounda' and 'Tassaout') were studied. Seeds were obtained from the Guich Station of the National Institute of Agronomical Research (INRA) in Rabat.

Spikes were cut when microspores were in the binucleate stage and were submitted to a cold pre-treatment at 5°C during 10 days. In a sterile laminar flow, wheat spikes with beards cut were disinfected in 10% commercial sodium hypochlorite for 10 minutes then rinsed 2 or 3 times in sterile distilled water. After elimination of outer layers, the ovaries were placed in ste-

rile petri dishes containing the culture medium solidified with 0.8% agar with the pH adjusted to 5.8 before autoclaving.

In the first experiment using an MS (Murashige and Skoog 1962) medium with 2 mg.L^{-1} of 2,4-D, four concentrations of sucrose were tested (45, 60, 90 and 120 g.L^{-1}. In the second experiment, using an MS medium with 120 g. L^{-1} of sucrose, three concentrations of 2,4-D were employed (2, 3.5 and 5 mg L^{-1}). In the last, series of (A, B, C) were conducted on two different media MS and C17 (Wang and Chen 1993) for study interaction between sucrose concentration and 2,4-D hormone quantities (Table 1).

Table 1 : Various combination of concentrations sucrose/ 2,4-D

		Sucrose (g L^{-1})			
2,4-D(mg.l^{-1})		45	60	90	120
	2 (A)	A1	A2	A3	A4
	3.5 (B)	B1	B2	B3	B4
	5 (C)	C1	C2	C3	C4

Ovary cultures were placed in darkness at 24± 2°C until callus formed in some cases. Calli were transferred to an MS regeneration medium without regulatory substances and maintained in a "16h photoperiod until shoots appeared. They were later transferred to a modified R9 medium containing 1 mg L^{-1}" of both IAA (indoleacetic acid) and kinetin.

Percentages of callus induction (% I) and regeneration (% R) were expressed for the total number of ovaries. In view of the low regeneration rate, the statistical study was limited to the induction phase. For each factor separately studied, the comparison of responses among different cultivars and different treatments was done using the analysis of variance according to the Newman and Keuls test at 0.5% threshold.

Results and discussion :

1. Influence of sucrose concentration on callus induction

With 2 mg L^{-1} of 2,4-D in an MS basal medium (Fig. 1), there was a marked effect of sucrose concentration on the percentage of induction. No callus was obtained with 45 g $L^{-1}$of sucrose for all cultivars, whereas for higher concentrations, responses varied with the cultivars. Thus for 'Jawhar', 'Belbachir' and 'Tassaout', induction took place at concentrations of 60 g L^{-1} whereas for 'Anouar', 'Sebou', 'Jori' and 'Kyperounda', induction requires 120 g L^{-1} of sucrose. For

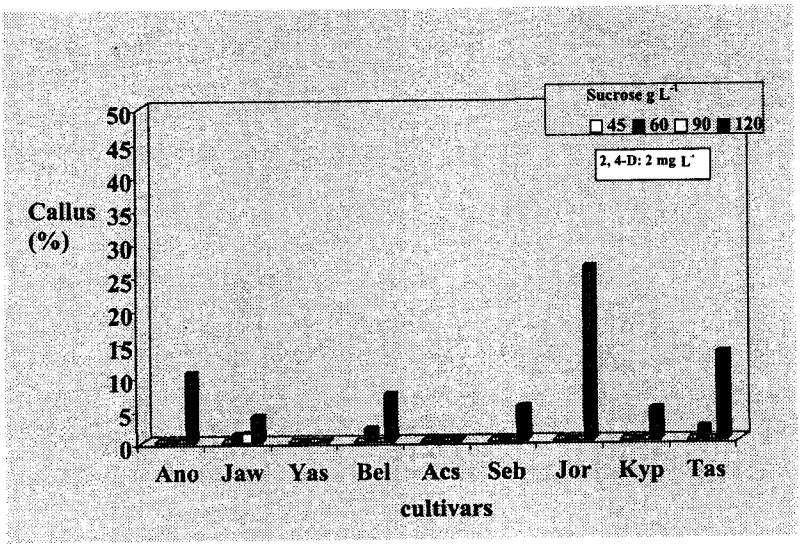


Figure 1 : Effect of sucrose concentration on callus induction on MS medium

'Yassmine' and 'Acsad65', no response was observed for all tested concentrations. The analysis of the variance of calli induction, according to the test of Newman and Keuls showed a significant difference due to interactions between sucrose concentrations and cultivars (Table 2)

Table 2 : Variance analysis effect of cultivar, sucrose, 2,4-D and their interactions according to the test of Newman and Keuls

Sources	df	Sum squares	Means square	Values	Pr>F
Sucrose	3	8.17	2.72	76.87	0.0001***
Cultivars	8	3.49	0.43	12.30	0.0001***
2,4-D	2	2.14	1.07	30.22	0.0001***
Sucrose/cultivar	24	5.87	0.24	6.90	0.0001***
Sucrose/2.4-D	6	1.20	0.20	5.65	0.0001***
Cultivar /2.4-D	16	2.13	0.30	3.76	0.0001***
Sucrose/2,4D/cultivar	48	4.33	0.10	2.55	0.0001***

*** Highly significant Difference at 0.5%

2. Influence of 2,4-D concentration on callus induction

For a sucrose concentration of 12% in an MS basal medium, four types of genotypic response were distinguished with rising 2,4-D concentrations (Fig. 2). For 'Anouar', 'Jawhar' and 'Yassmine', callus induction rates rose with 2,4-D concentration. For 'Sebou' and 'Kyperounda', induction rates varied little. For 'Jori', 'Belbachir' and 'Tassaout', callus induction diminished with rising 2,4-D concentration and for 'Acsad65', no response was obtained in all cases.

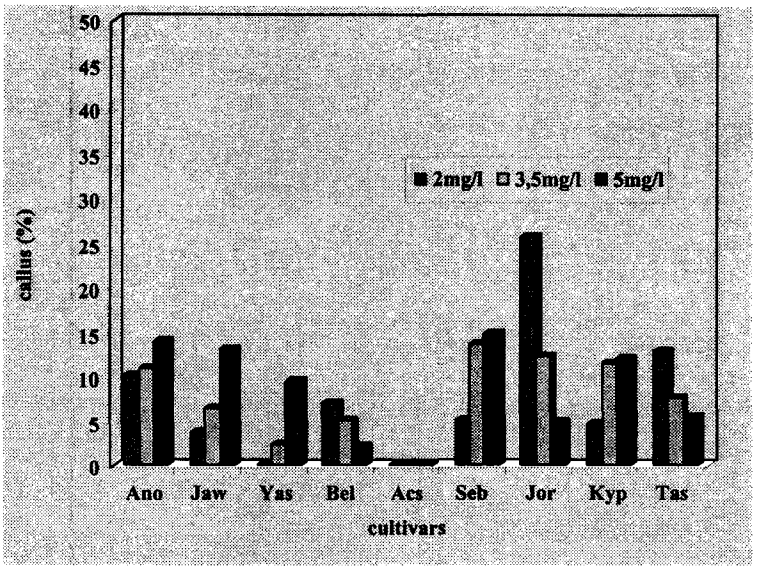


Figure 2 : Effect of 2,4-D on callus inductionon MS medium

These results could be explained by an interaction between 2,4-D concentrations and the cultivars and this has been shown statistically with the analyse of the variance according to the test of Newman and Keuls (Table 3).

Table 3: Effect of two basal media MS and C17 with various Concentrations of 2,4-D and sucrose

	A2	A3	A4	B3		B4	C2		C3		C4	
	MS	C17	MS	MS	C17	MS	MS	C17	MS	C17	MS	C17
Tassaout	1.85	0.00	13.04	2.22	0.00	0.00	0.00	13.33	0.00	4.25	5.48	0.00
Kyperounda	0.00	0.00	4.76	0.00	0.00	0.00	0.00	17.74	8.33	0.00	12.3	0.00
Jori	0.00	0.00	25.8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.9	0.00
Sebou	0.00	0.00	5.09	2.12	0.00	0.00	0.00	22.39	2.22	0.00	15.01	5.97
Belbachir	1.85	0.00	7.14	0.00	2.00	0.00	9.30	38.77	6.38	17.02	2.01	0.00
Yassmine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.90	3.45	2.8	9.55	2.78
Jawhar	0.00	1.30	3.79	3.44	1.49	1.29	4.59	23.07	7.95	11.49	13.18	7.89
Anouar	0.00	0.00	10.26	3.3	2.85	1.28	0.00	0.00	0.00	0.00	0.00	0.00

NB: media wich gave no response were omitted)

3-Action of two basal media (MS, C17) with various concentrations of 2,4-D and sucrose

Apart from conditions A1, B1 and C1 (where sucrose is at 45 $g.L^{-1}$), and where no callus induction is observed, a marked effect of the two basal media was observed. MS compared with C17 (Fig. 3) gave higher induction rates varying from 0.35% for A3 to 16.5% for C4. For MS, the best response in each series was obtained with 12% sucrose: 16% for series A, 13% for series B and 16.3% for series C. This high level of sucrose might provide an osmotic pres-

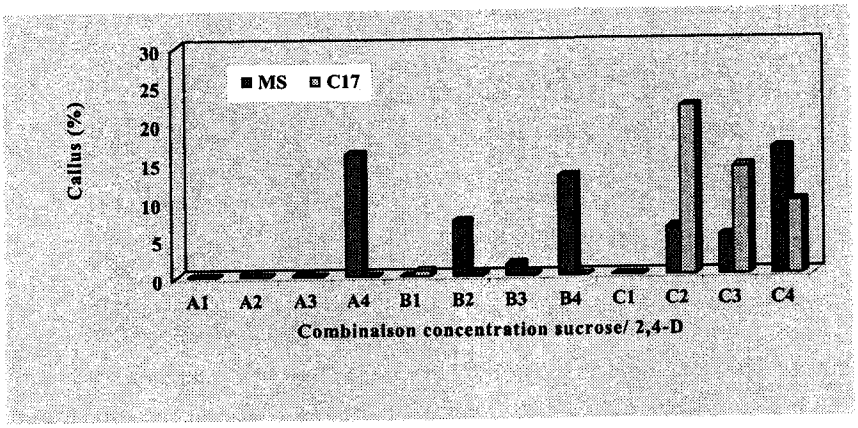


Figure 3 : Combined effect of sucrose and 2,4-D on MS and C17 media

sure favorable for in vitro embryo formation as has been shown in Brassica (Kameya and Hinata 1970) and Petunia (Wakizuka and Nakajima 1974) ovary cultures and wheat anther cultures.

For C17 medium, no response was observed for the A series (2 mg L^{-1} of 2,4-D) and only 5% induction in series B (3.5 mg L^{-1} of 2,4-D). A clear increase was observed for the series C (5 mg.L^{-1} 2,4-D) with an optimum of 21.9% for C2.

Table 3 shows a differential response of cultivars to the culture media. For MS, the three cultivars with highest responses were 'Jori', 'Tassaout' and 'Anouar' whereas for C17, the best three were 'Belbachir', 'Anouar' and 'Jawhar'. The analysis of variance according to the test of Newman and Keuls was made with the media as the source of variation showed a significant difference at 0.5% (Table 4).

Table 4: Variance analysis with the media as source of variation

Sources	df	Mean squares	Values	Pr>F
media	1	0.43154307	31.94	0.0001***

*** Highly significant Difference at 0.5%

4. REGENERATION

After transfer of calli to regeneration medium containing no regulatory substances in a 16 h photoperiod, shoots were formed in some cases (Fig 4a, 4b and 4c). Shoots and callus were then transferred to R9 medium with 1 mg.L^{-1} of both IAA and kinetin which promoted the development of shoots into young plants.

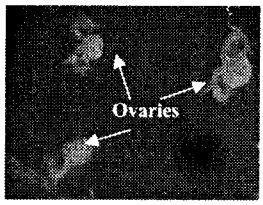
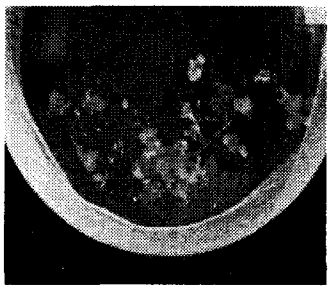


Figure 4a : Ovaries culture



1 : Ovary; 2 : callus; 3 : regeneration and 4 : roots

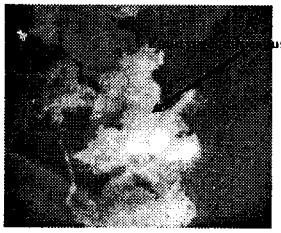


Figure 4b : Embryogenesis callus initiated

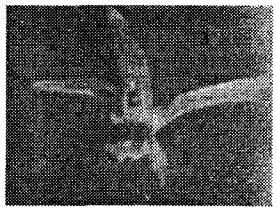


Figure 4c : Green plant differentiation

Wide genotypic differences were observed in regeneration rates for the various cultivars. Seventy per-cent of all regenerations involved 'Jori', 'Anouar' and 'Jawhar'. Two cultivars ('Yassmine' and 'Kyperounda') gave no regenerations (Table 5).

The aptitude for regeneration (% R) seems to be independent of the induction capacity: the variety Belbachie' with a 13.10% induction showed 4.8% regeneration, while 'Jawhar' with a %I of 9.56% had a 6.6%R. As for 'Kyperounda' and 'Yassmine', with %I rates respectively of 5.53% and 7.35%, no regeneration was observed. The independence of these two traits has already been shown for androgenesis in durum wheat (Saidi et al. 1997) and bread wheat (Picard et al 1994) and suggests that they are controlled by independent genes. The regenerate plants are supposed to be haploid, it is based on a chromosome counting of a root sample that showed a chromosome number n=14. On the other hand, origin of callus formation can be supposed to be haploid: callus induction takes place at the ovule level (haploid).

Sixty nine regenerations were obtained during these experiments. All regenerated plants were chlorophyllous, in marked contrast with haploid regeneration through androgenesis in durum wheat.

Table 5: Percentage of plant regeneration from unpollinated ovarie cultures

	ovaries	Callus induction		Regeneration	
		Number	%	Number	%
Tassaout	231	17	7.35	4	1.73
Kyperounda	253	14	5.53	0	0
Jori	239	14	5.85	12	5.02
Sebou	202	8	4.45	5	2.47
Belbachir	145	19	13.10	7	4.8
Yassmine	2 87	21	7.31	0	0
Jawhar	209	20	9.56	14	6.69
Anouar	378	34	8.99	27	7.14

CONCLUSIONS

Based on our experiences, we could conclude that gynogenetic capacity is conditioned by some key factors: high concentration of sucrose is decisive for some cultivar, on the other hand, the amount of the 2,4-D has an important action in the induction phase. These results showed also that cultivar with the highest for callus induction potential do not necessarily present the best aptitude. This is in agreement with durum wheat androgenesis results (Picard et al 1994).

Our major conclusion could be that the C17 medium added with $60g.L^{-1}$ and $5mg.L^{-1}$ (C17, C2) is the most adapted to ovary culture (in this experience). On another hand, with this medium, in the most cultivars, callus induction has been observed and the best one was Belbachir (37.77% induction).

The callus origin is supposed to be from ovules, the histological study is considered to confirm Assertion. Regenerated plant haploid is deduced from two observations; the first one is based on preliminary chromosome counts in some root tips of regeneration plant, the second one, the way callus regenerate; they appeared in an opening at the base of the ovary. The ovarian walls fall of progressively and the callus is then able to grow by it self.

The good regeneration yield of gynogenesis would make it a valid alternative for producing durum wheat double haploid lines.

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