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Original Paper

Efficient callogenesis and plant regeneration in bread wheat (*Triticum aestivum* L.) varieties

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Five bread wheat varieties (*Triticum aestivum* L.) were evaluated *in vitro* by culturing explants of scrapped mature embryos (ME) and endosperm-supported mature embryos (MES) on Murashige and Skoog (MS) medium fortified with 2, 3, and 4 mg.l^{-1} of Dichlorophenoxyaceticacid (2,4-D) for callogenesis and proliferation. The regeneration was initiated first, on MS hormone-free and then continued on MS complemented with indoleacetic acid (IAA) and 6-benzyladenine (BAP). 2 mg.L^{-1} 2,4-D was found to be optimum for callus induction and embryogenic callus production. As for plant regeneration, all five varieties have been able to form shoots and roots. However, this parameter was strongly controlled by variety and explant type. The highest percentages of regeneration were established at 80% for the endosperm-supported mature embryos and 68% for mature embryos. The success of any morphogenesis *in vitro* culture results from a better optimization of culture conditions (mineral and hormonal composition of the medium, explant type, and callus age).

Keywords: explant type, phyto-regulator, embryogenic callus, *in vitro* culture

1 Introduction

Cereal crops occupy a key position in agricultural research programs on a global basis. Wheat is a plant of the grass family Poaceae, belonging to the genus *Triticum*. Bread wheat known as *Triticum aestivum* L. is a hexaploid species commonly referred to as "common" or "soft". The majority of commercial breads marketed worldwide are made from T. aestivum ssp., which are distinguished by naked kernels, advantageous for milling in bread and pasta preparation, as well as in baking (Martín-Gómez et al., 2019). It has long been recognized as the cereal king. Because of its worldwide production and marketing, it provides humanity with important nutrients. The continual growth in population and loss of agricultural areas due to sustainable urbanization, along with negatively changing environment, poses major concerns to the secured production of wheat (Al-Ashkar et al., 2022). With the world's population predicted to reach 9.6 billion by 2050, consumption for main cereals such as wheat, rice, and maize might hit 3.3 billion tons per year. As a result, supplies must continue to rise by 2–3% every year to fulfil the requirements of the expanding population (Al-Ashkar et al., 2022). For growing crops, recent scientific advances have been investigated in order to provide a diverse range of choices and novelties in plant breeding. Several researchers have investigated yield enhancement in order to generate plants with desirable features (Ahmar et al., 2020). Over the years, *in vitro* plant cell culture has attracted particular interest because it offers opportunities to explore the physiological and genetic mechanisms of plants, as well as, contribute to the screening of varieties with improved characteristics by enhancing genetic variability. These techniques have gained importance in wheat breeding. Thus, the use of various methods in cereals enhancement implicates the establishment of an efficient and consistent system. Indeed, one of the best biotechnological techniques

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applied for wheat crop development is the plant regeneration system (Munir, 2009). Plant regeneration has been defined by Keresa et al. (2001) as the primordial phase in the transformation of cereal crops. It has been set up in cell culture programs with various degrees of success starting from the callus induction stage.

Bread wheat (*Triticum aestivum* L.) is one of the most challenging cereals to *in vitro* culture, according to Razzaq et al. (2011), Additionally, as mentioned by Malik et al. (2021) Agrobacterium-mediated transformation and the gene gun, which involves the callogenesis step, have been used in previous and current investigations on wheat genetic transformation. The emphasis, nonetheless, is not gene delivery, but rather the establishment of a long-term culture and regeneration of transformed cell lines. For every plant tissue culture procedure, induction of embryogenic callus and regeneration of transformed plants are necessarily controlled by many interesting factors. Among these, Kumar et al. (2017), and Hakam et al. (2014) have all stated the importance of selecting potentially more regenerative genotypes, using the most efficient explants. Other researchers such as Mahmood et al. (2012) and Parmar et al. (2012) mentioned in their work the effect of medium composition, hormonal balance, and conditions required for *in vitro* culture.

Auxins such as Dichlorophenoxyaceticacid (2,4-D), Dicamba, Picloram... etc. have been reported previously by Mahmood et al. (2013), and Hakam et al. (2015). They evaluated them at different dosages to induce and form callus with embryogenic potential. On the other hand, for plant regeneration Yu et al. (2008) have reported in their work the effective usage of auxins such naphthalene acetic acid (NAA) and indole acetic acid (IAA) in combination with cytokinins like zeatin, kinetin, and 6-benzyladenine (6-BA). In addition, all approaches in tissue culture require the choice of a favourable explant and genotype. In this manner, variable responses for callogenesis and vitro-plants regeneration were described in many studies. For instance, immature embryos tested by Hakam et al. (2015) and Ahmadpour el al. (2016), leaf tissue explant experienced by Yu et al. (2008), and mature embryos explored by Hasanuzzaman et al. (2021), and various other types were all chosen as the initial source of explants for the *in vitro* experiment. As previously reported by Yang et al. (2015), immature embryos are primarily the preferred source of explants for wheat *in vitro* culture, because of their high callus induction and regenerative capacity. However, their availability and productivity are limited throughout the year and it is difficult to culture and produce during the off-season. Conversely, explants, such as mature seeds, excised mature embryos, and endosperm-supported mature embryos were tested by Aydin et al. (2011) in their wheat tissue culture studies. These explants are easy to conserve and available throughout the year. Nevertheless, the low callogenesis rate and regeneration potential of mature embryos remains a major defect that prevent researchers from using them in practice (Tamimi & Othman, 2021). Hence, the adoption of a promising explant and the optimization of a reliable regeneration protocol starting from the callogenesis stage may overcome these limitations. In this study, we aim to settle a simple yet, effective *in vitro* culture protocol for the disinfection of explants, callogenesis, embryogenic callus formation, and their regeneration in five wheat varieties cultivated in Morocco, based on the optimization of some factors controlling *in vitro* culture (explant types, hormonal composition and incubation period).

2 Material and methods

2.1 Plant material and seeds sterilization

The seeds were supplied by the national office of food safety – ONSSA-Rabat. Five bread wheat varieties (Wissam, Nassim, Wafia, Rajae, and Tigre) were studied. These varieties are listed in the catalog of SONACOS "National Seed Marketing Corporation – Morocco" (Table 1). The experimentations were conducted at the "Agro-Physiology, Biotechnology & Environment" Research Unit, Laboratory of Natural Resources & Sustainable Development, Ibn Tofail University, Faculty of Science, life sciences Department, Kenitra, Morocco.

The mature seeds of the five wheat varieties were rinsed several times with Lukewarm distilled water after being washed with tap water containing a few drops of Tween 20. After being soaked in sterile distilled water overnight at room temperature, the seeds were disinfected by soaking and high shaking in 45% of commercial bleach (sodium hypochlorite) for 20 minutes, then rinsed six times with sterile distilled water with continuous shaking in horizontal laminar flow hood, the seeds were then, dried with sterile filter paper in sterile Petri dishes.

2.2 Explants preparation for callus induction

Two types of explants of the five wheat varieties were used, which are endosperm-supported mature embryo (MES) and mature embryos (ME).

2.2.1 Endosperm-supported mature embryo (MES)

In a Petri dish, sterilized seeds were placed on sterile filter papers, the embryos were half removed (still attached to the seeds) and then several wounds with the aid of a sharp scalpel, were made at the level of the embryonic axis and

Table 1 Principal characteristics of five wheat varieties grown in Morocco

Variety	Provenance and date of inscription	Main characteristics
NASSIM	Semillas Batlle – Spain (2000)	the plant is of average size, the seeds are of white color; cycle at maturity: half-late productivity: very high; weight of 1,000 grains: 29 to 31g (very moderate) the culture of this variety is recommended for the bour regions and irrigated
WAFIA	Desprez – France (2005)	the size of the plant is average, the seeds are of red color; cycle at maturity: half-early productivity: very high; weight in 1,000 grains: 41 to 44 g this variety is characterized by a very high productivity
RAJAE	INRA – Morocco (1993)	height of the plant: 70 to 105 cm according to the conditions; cycle at maturity: half early productivity: high; weight in 1,000 grains: 30 to 34 g the culture of this variety is recommended for the sub-humid regions and irrigated
TIGRE	INRA – France (1996)	plant height: medium; maturity cycle: semi-early productivity: very high; weight in 1,000 grains: 30 to 34 g this variety is recommended for allocated to irrigated land and to the best bour plots with deep soils
WISSAM	ETS Maire des fontaines-France(1995)	of high size, the seeds are of ginger color; cycle at maturity: semi-precocious productivity: excellent; weight of 1,000 grains: 50 to 52g

Source: Andaloussi & Chahbar, 2005; Aït Houssa et al., 2016; SONACOS, n. d.

different sides of the seed's embryo. The explants were placed in the sterile Petri dishes containing the induction medium.

2.2.2 Mature embryos (ME)

Under aseptic conditioning, mature embryos were entirely excised from sterilized seeds. This is done by placing a small hole between the top of the embryo and the endosperm then gently and completely detaching it from the rest of the seed. The endosperm separated from the embryo can then be discarded. During this procedure, we made sure that proper and sterile equipment, namely scalpels and forceps, are used to properly excise the embryo. The embryos were then scraped gently and fully without causing any damage. The explants were placed afterwards on medium culture facing the scutellum side up.

Both techniques are used (lesions and scraping) in order to improve both the entry of the growth regulator and the inhibition of explant germination.

2.3 Callogenesis induction

For callus induction, three media were investigated, The basal culture media consisted of the mineral salts of MS (Murashige and Skoog, 1962) supplemented with 2, 3 and 4 mg.l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). All media contained 30 g.l^{-1} sucrose and 0.1 g.l^{-1} Myo-inositol and were adjusted to pH 5.7, solidified with 8 g.l^{-1} agar and autoclaved at 120 °C for 20 min., at 1 bar pressure. For this test, 30 replications were used for each type of explant (ME or MES) representing a total of 1,500 explants (5 explants per 10 Petri dishes per 3 concentrations of 2,4-D per each of the five varieties per each of the 2 types of explants (ME or MESs). The incubation was conducted in the dark at 25 °C for three weeks. After 10 days of callogenesis, unwanted tissues were removed with a sharp sterilized scalpel under aseptic conditions. The cleared calli were then sub-cultured for another 11 days on new medium culture with the same composition and the same 2,4-D concentrations (2; 3 and 4 mg.l^{-1}). The aspect of induced callus and callogenesis rate were determined according to the following formula:

$$C\% = \left(\frac{number\ of\ induced\ calli}{total\ number\ of\ explants} \right) \times 100$$

At the end of the callogenesis phase, 300 calli, including 150 calli per type of explant (10 calli per 3 concentration of 2,4-D per 5 varieties) were individually weighed on an electric balance to record their masses after being dried in an oven at 75 °C for 48 hours. The dry materials were taken in mg.

2.4 Proliferation of calli

To distinguish between embryogenic calli and non embryogenic calli, the 21 days old induced calli from both types of explants were cleaned from any unwanted parts and maintained on fresh MS media supplemented with the same hormonal composition used at callogenesis stage (2, 3 and 4 mg.l^{-1} 2,4-D). Calli were shifted for one week into a grow room at 25 °C under a photoperiod.

2.5 Regeneration

The calli distinguished as embryogenic were cut into small fractions at an amount of 750 calli (5 fractions X 15 Petri dishes X 1 medium X 5 varieties X 2 types of explants), then transferred on regeneration initiation MS media without growth regulators. To stimulate further development after 24 days, calli were then shifted on new

fresh media MS supplemented with 0.1 mg.l^{-1} AIA + 0.5 mg.l^{-1} BAP. The incubation was conducted at 25 ±1 °C under photoperiod of 16 hours of light in grow room using cool white fluorescent tubes at approximately 2,000-lux light intensity for illumination. The regeneration rates were calculated after a total of 8 weeks according to the following formula:

$$(R\%) = \left(\frac{number\ of\ regenerated\ calli}{total\ number\ of\ cultured\ calli} \right) \times 100$$

2.6 Statistical analysis of data

Analysis of variance (ANOVA) was used to assess the effect of hormone concentration, variety, type of explant, and their interaction on different parameters. The Shapiro-Wilk test was used to determine the normality of the data distribution. If necessary, the normality was filled by transformation. The comparison of means was performed using the Tukey HSD (Honestly Significant Difference) test with a risk of error of 0.05. Data Analysis was conducted by JMP SAS Pro software (JMP®, Version <14> SAS Institute Inc.).

3 Results and discussion

Five varieties of wheat were evaluated on three types of induction media. The first step was callus induction from both types of explant. After that, wheat plants could be regenerated from embryogenic callus.

3.1 Callus induction

3.1.1 Types of induced calli

For all varieties tested, the two types of explants begin to form callus after 3 days of culture (Figure 1). The callus's colour was off-white or pale yellowish, have a compact nodular or rough texture (A), while the other type of callus are transparent in color, shows a soft-loose consistency and watery texture (B).

3.1.2 Callus initiation rate (%)

An excellent callogenesis rate was observed (Figure 2) as well for ME (between 100% and 80%) as for MES (between 100% and 86%) used as two types of explants on the Medias supplemented with the three concentrations of 2,4-D (2, 3 and 4 mg.l^{-1}). Statistical analysis shows a significant effect of different 2,4-D levels, the variety as well as the interaction between the type of explant

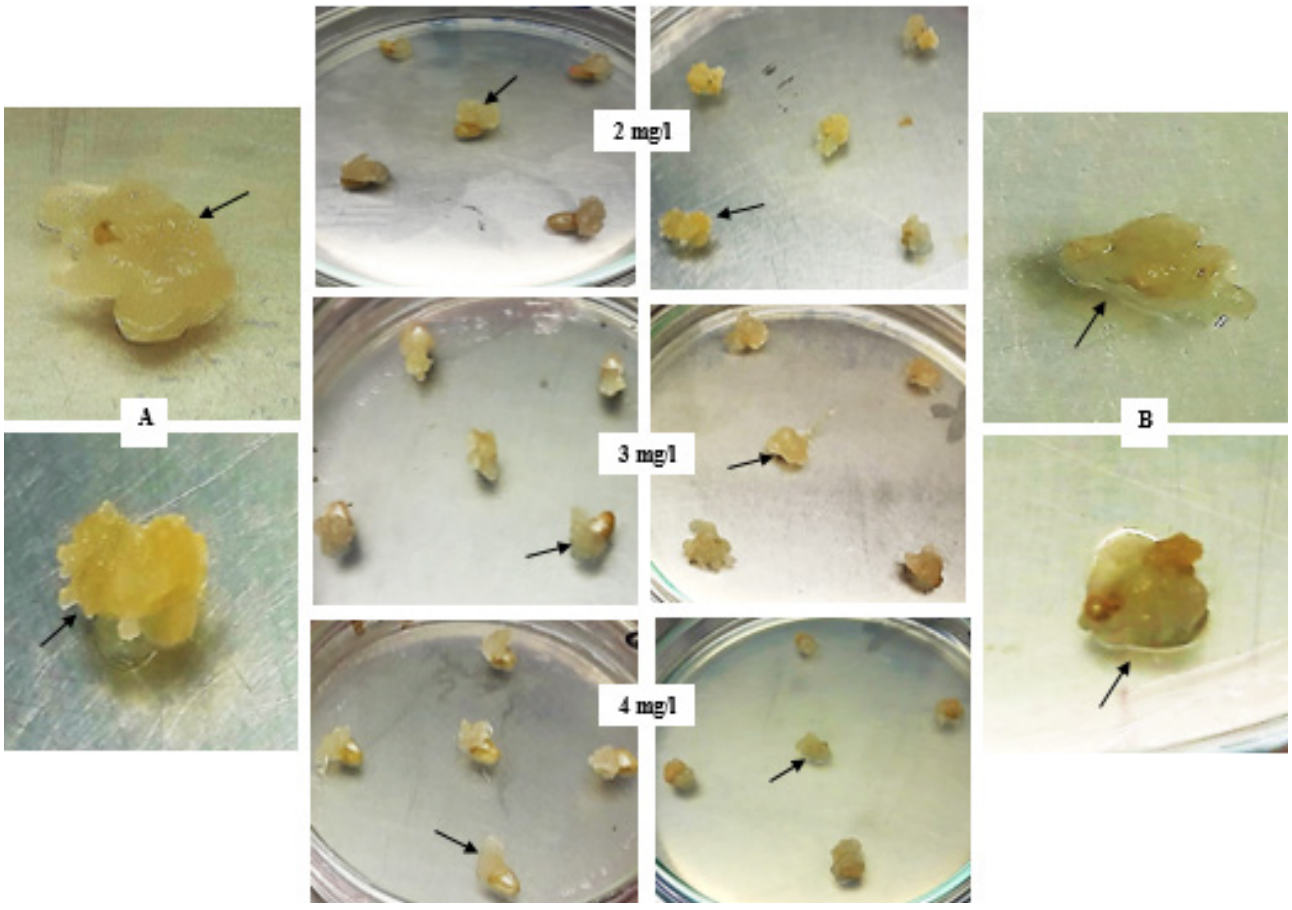

Figure 1 Callogenesis induction from endosperm-supported mature embryos and mature embryos depending on hormonal concentration; A and B –aspect of callus obtained after three weeks of culture

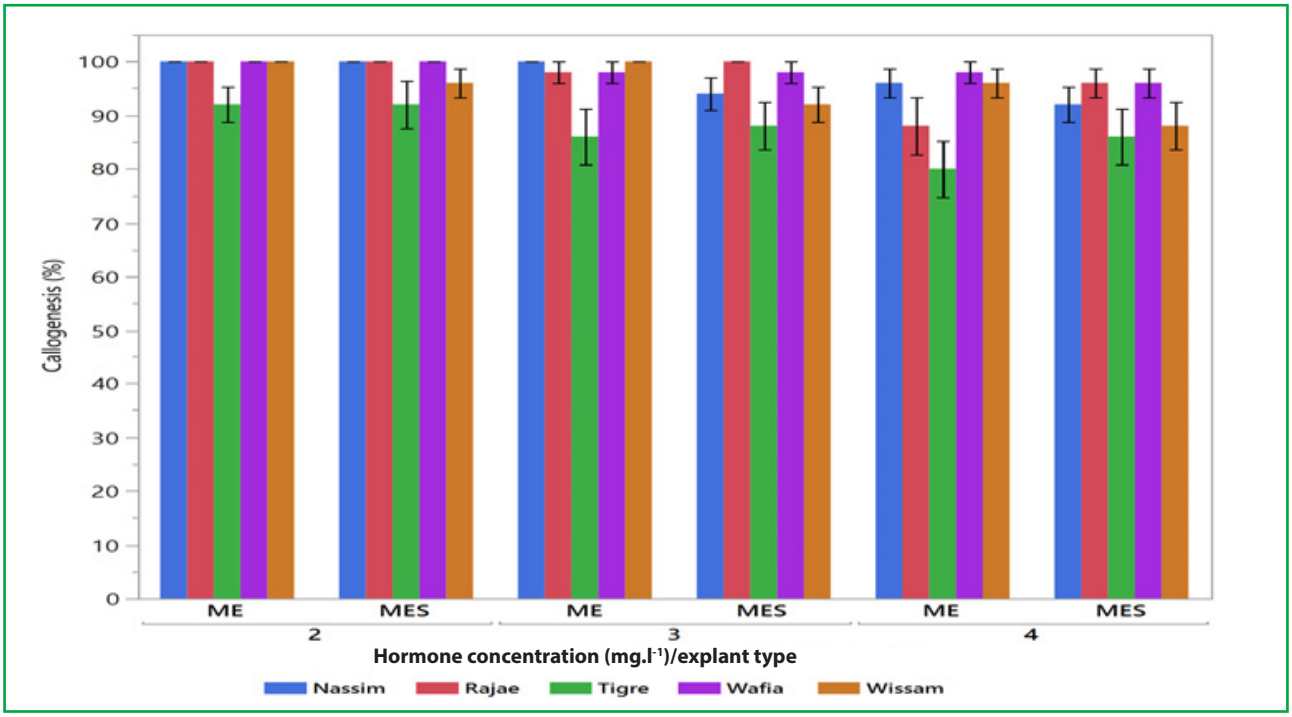

Figure 2 Effect of different hormone concentration and explant type on callogenesis of five varieties in wheat
data presented as mean (±SE)

and the variety on this parameter (Table 2). According to the media effect, it applied a highly significant effect on callogenesis. However, 2 et 3 mg.l^{-1} were better than 4 mg.l^{-1} by showing the best response in all tested varieties (Table 3). The Varieties' response was variable. Indeed, the ranking of means by Tukey test shows two groups for calli derived from mature Embryo ({Nassim = Wissam = Rajae = Wafia}> {Tigre}). As for the comparison of the explants, the statistical analysis shows no significant differences between the means (Table 4).

3.2 Mass of dry matter (mg)

Figure 3 illustrates the recorded masses of dry matter (DM) for the five wheat varieties. Overall, this parameter was variable according to the hormonal concentration, variety factor. However, for both type of explants, the variable DM depends only on the hormonal concentration (Table 2). The concentration of 2 mg.l^{-1} appears more suitable compared to 3 and 4 mg.l^{-1}. At the concentration 2, 3 and 4 mg.l^{-1} 2,4-D, the masses recorded by the calluses from MES are (15.9; 9; 8.98 mg), (13.1; 11.5;

Table 2 ANOVA results to test the effects of different levels of 2,4-D and the type of explant (ME/MES) as well as their interaction on callogenesis, fresh and dry matter, and the percentage of embryogenic calli of five wheat varieties

Sum of squares			
Source of variation	DF	callogenesis (%)	dry matter (mg)
Type of explant (E)	1	65.33^{ns}	55.99^{ns}
Variety (V)	4	4,680.00***	198.46*
E × V	4	1,048.00*	13.53^{ns}
Concentration (C)	2	2,072.00***	991.83***
E × C	2	50.67^{ns}	25.71^{ns}
V × C	8	328.00^{ns}	403.92**
E × V × C	8	376.00^{ns}	39.60^{ns}
Model	19	8,620.00***	1,729.04***
Error	40	23,480.00	4704.21
C. Total	59	32,100.00	6,433.25

DF – degree of freedom; ns – not significant; *, **, *** significant at 5%, 1% and 0.1% level

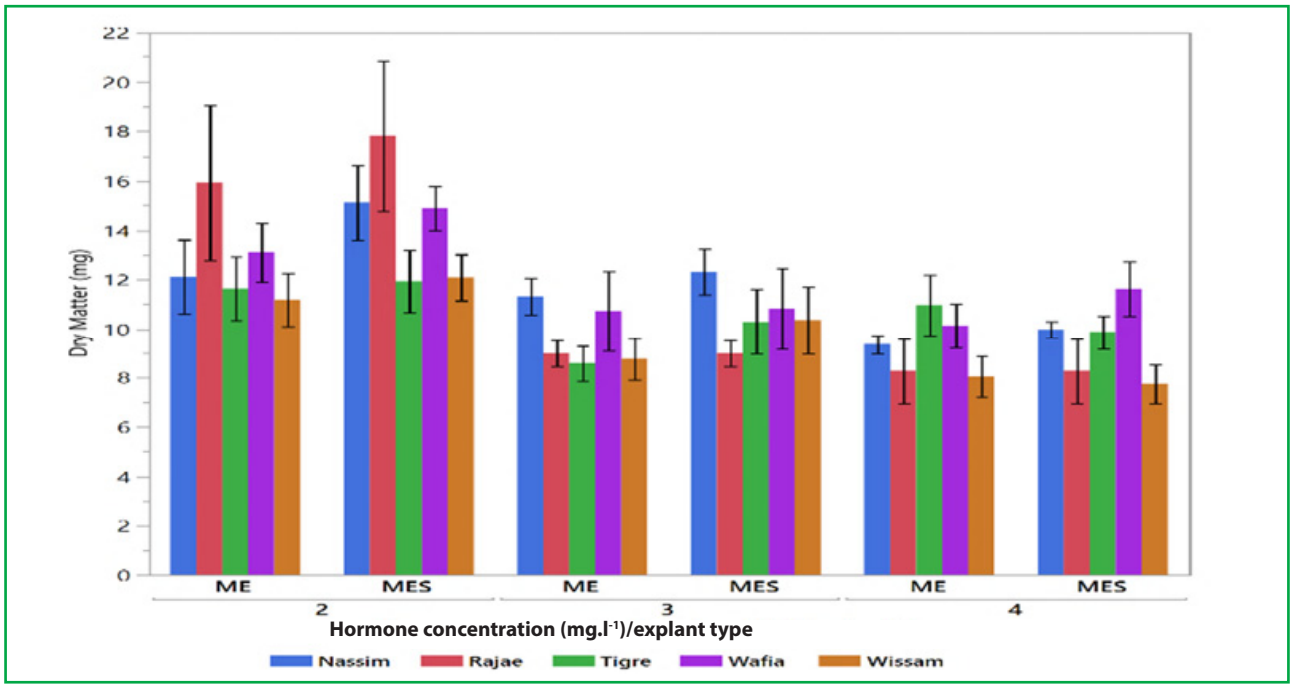

Figure 3 Effect of different hormone concentration and explant type on dry matter of five varieties in wheat
data presented as mean (±SE)

10.1 mg), (12.1; 11.3; 9.37 mg), (11.6; 8.59; 11 mg) and (11.2; 8.27; 9 mg) respectively, in Rajae, Wafia, Nassim, Tigre and Wissam. Statistical analysis of variance (Table 2) showed that the variable (DM) is significantly depending on the hormonal concentration, the variety and their interaction. Whereas, the differences for the interactions for the "variety x hormonal concentration" factors are very significant. Regarding the result, the concentration of 2 mg.l^{-1} 2,4-D seems to be more appropriate compared to 3 and 4 mg.l^{-1} (Table 3). Comparison of the means for the variable MS shows two groups for the variety factor ({Wafia} ≥{Nassim = Rajae = Tigre} ≥{Wissam}) and two groups ({2} >{3 and 4} mg.l^{-1} 2,4-D) according to the hormonal concentration factor. However, for the two types of explants, the differences noted for the variable (MS) are not significant (Table 4).

3.3 *Calli proliferation*

3.3.1 *Type of embryogenic calli*

For both types of explants, calli were distinguished as embryogenic callus (EC) and non-embryogenic callus (NEC). Embryogenic callus (A) were characterized by and off-white to pale yellowish colour, compact in consistency. They are containing fused nodules that start to form some green spots (B) at the end of the proliferation phase; While, non-embryogenic calli {(C), (D)} were transparent, soft-loose and mucilaginous in consistency and watery in texture, incapable

Table 3 Comparison of means according to the effect of different 2,4-D levels, variety and their interaction on different parameters

Concentration (mg.l^{-1})	Callogenesis (%)	Dry matter (mg)
2	98.00 ±0.67^{a}	13.59 ±0.57^{a}
3	95.40 ±0.98^{a}	10.12 ±0.35^{b}
4	91.60 ±1.28^{b}	9.43 ±0.31^{b}
Variety		
Nassim	97.00 ±0.93^{a}	11.70 ±0.47^{ab}
Rajae	97.00 ±1.15^{a}	11.38 ±0.92^{ab}
Tigre	87.33 ±1.90^{b}	10.55 ±0.46^{ab}
Wafia	98.33 ±0.72^{a}	11.89 ±0.54^{a}
Wissam	95.33 ±1.20^{a}	9.70 ±0.44^{b}

the data are presented by means (±SE); values (mean ±SE) followed by different superscripts letters are significantly different at $p = 0.05$

Table 4 Comparison of means according to the effect of different 2,4-D levels, variety and type of explant on different parameters

2,4-D (mg.l^{-1})	Variety	Callogenesis (%)		Dry matter (mg)	
		explant type			
		ME	MES	ME	MES
2	Nassim	100.00^{a} ±0.0	100.00^{a} ±0.00	12.12^{abcd} ±1.50	15.12^{abc} ±1.52
	Rajae	100.00^{a} ±0.0	100.00^{a} ±0.00	15.92^{ab} ±3.14	17.82^{a} ±3.04
	Tigre	92.00^{ab} ±3.27	92.00^{ab} ±4.42	11.64^{abcd} ±1.29	11.94^{abcd} ±1.28
	Wafia	100.00^{a} ±0.00	100.00^{a} ±0.00	13.12^{abcd} ±1.19	14.89^{abc} ±0.89
	Wissam	100.00^{a} ±0.00	96.00^{a} ±2.67	11.19^{abcd} ±1.09	12.09^{abd} ±0.94
3	Nassim	100.00^{a} ±0.00	94.00^{ab} ±3.06	11.32^{abcd} ±0.75	12.32^{abcd} ±0.92
	Rajae	98.00^{a} ±2.00	100.00^{a} ±0.00	9.00^{bcd} ±0.53	9.00^{bcd} ±0.53
	Tigre	86.00^{ab} ±5.21	88.00^{ab} ±4.42	8.59^{cd} ±0.71	10.29^{bcd} ±1.32
	Wafia	98.00^{a} ±2.00	98.00^{a} ±2.00	10.72^{bcd} ±1.61	10.82^{abcd} ±1.64
	Wissam	100.00^{a} ±0.00	92.00^{ab} ±3.27	8.77^{bcd} ±0.84	10.36^{bcd} ±1.36
4	Nassim	96.00^{a} ±2.67	92.00^{ab} ±3.27	9.37^{bcd} ±0.37	9.97^{bcd} ±0.34
	Rajae	88.00^{ab} ±5.33	96.00^{a} ±2.67	8.28^{cd} ±1.31	8.28^{cd} ±1.31
	Tigre	80.00^{b} ±5.16	86.00^{ab} ±5.21	10.96^{abcd} ±1.23	9.87^{bcd} ±0.67
	Wafia	98.00^{a} ±2.00	96.00^{a} ±2.67	10.13^{bcd} ±0.88	11.63^{abcd} ±1.10
	Wissam	96.00^{a} ±2.67	88.00^{ab} ±4.42	8.06^{cd} ±0.83	7.75^{d} ±0.79
Average		95.47^{a} ±0.85	95.47^{a} ±0.85	94.53^{a} ±0.84	10.61^{a} ±0.36

the data are presented by means (±SE); values (mean ±SE) followed by different superscripts letters are significantly different at $p = 0.05$

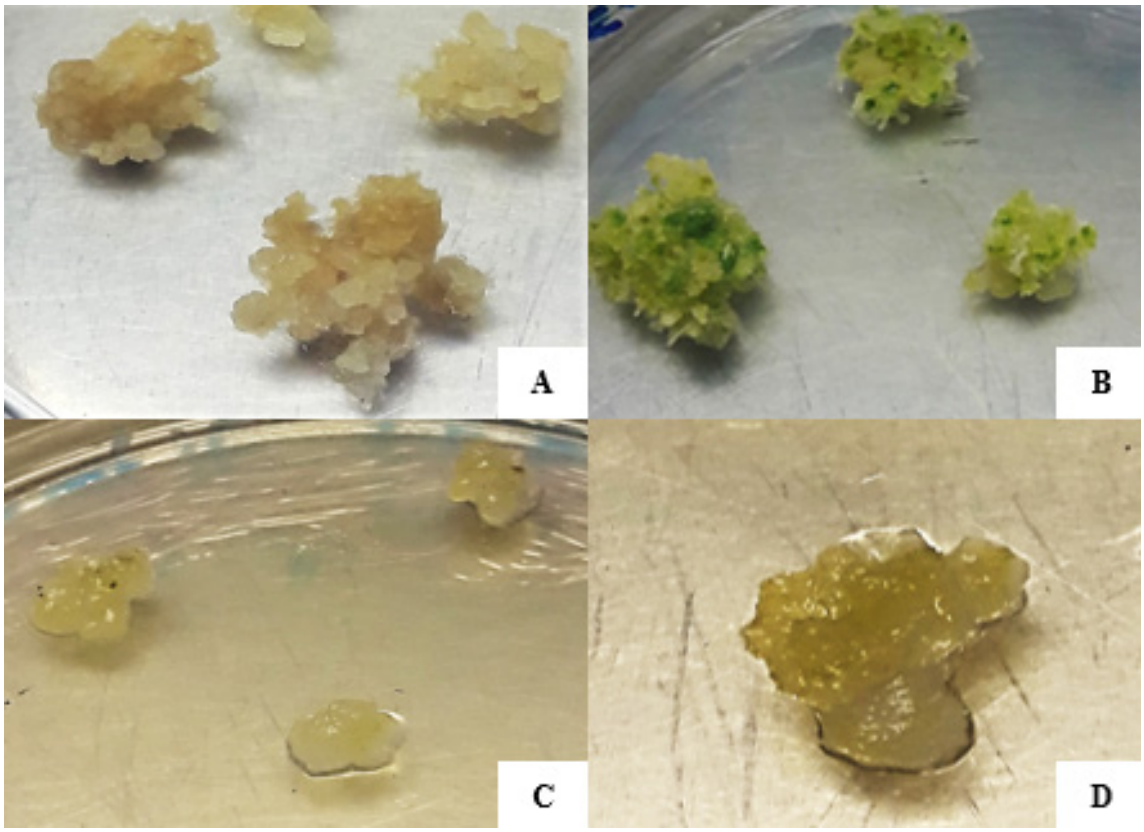

Figure 4 A and B – embryogenic callus and appearance of green spots at the embryogenic callus at the end of the proliferation; C and D – non-embryogenic callus

of producing somatic embryos regarding the period of incubation (Figure 4). Out of the three media tested, embryogenic calli favourably selected the 2,4-D medium at 2 mg.l^{-1} to be developed.

3.4 Regeneration

After one week of proliferation, embryogenic calli in media containing 2 mg.l^{-1} 2,4-D that were able to produce firstly shoots and then roots on the regeneration medium, were considered as responded embryogenic callus (Figure 5).

Plant regeneration proves successful with variable rates among all tested varieties (Figure 6). The use of the hormone-free MS medium favoured the formation of shoots and roots. However, the production rates were variable depending on all the varieties tested and the type of explant.

In the case of ME, maximum regeneration rates were noted, respectively, in Rajae varieties at 68% and Wafia at 66%, followed by Nassim varieties (50%). While Wissam and Tigre show values below 50%. On the other hand, for MES, the tested varieties appear to be more reactive with very high regeneration rates respectively, in Wafia (80%), Rajae (80%) and Nassim (73%). While Wissam and Tigre varieties recorded values of (59%) and (44%). Furthermore, regardless of the type of explant, calli proliferated for two and three weeks, showed no plant development. They eventually grew in volume and produced only roots. Therefore, their regeneration rate was considered null for both types of explants. Statistical analysis for this parameter revealed very highly significant differences for both the variety factor and the type of explant (Table 5). The compared means (Table 6) by the Tukey ranking for the varietal factor indicates three groups ({Rajae = Wafia})> {Nassim = Wissam} > {Tigre}). In accordance to the explant effect, calli from mature embryos supported by the endosperm act better at regeneration by producing more seedlings compared to those from mature embryos (Table 6).

Table 5 ANOVA results to test the effect of the type of explants (ME/MES) and the five wheat varieties as well as their interaction on regeneration

Sum of squares		
Source of variation	DF	Regeneration (%)
Type of explant (E)	1	6,666.67***
Variety (V)	4	24,624.00***
E *V	4	933.33^{ns}
Model	9	32,224.00***
Error	140	27,733.33
C. Total	149	5,9957.33

DF – degree of freedom; ns – not significant; *, **, *** significant at 5%, 1% and 0.1% level

In this work, we described some important criteria such as the impact of the 2,4-D concentration, age of callus, medium and variety control as major factors resulting in producing embryogenic callus and their regeneration

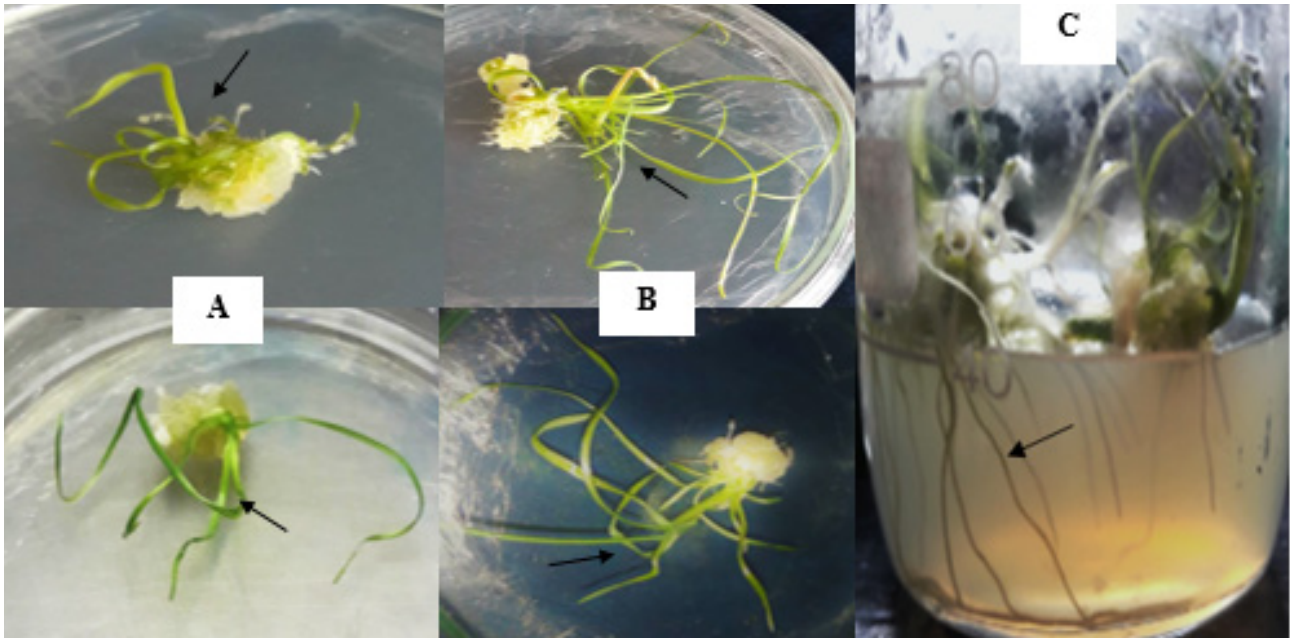

Figure 5 Regeneration of vitro-plants from regenerating callus of endosperm-supported mature embryos (A) and excised mature embryos (B) proliferated for one week. (C): Roots development of regenerated plants after transfer to Ms Medium 0.1 mg.l^{-1} AIA + 0.5 mg.l^{-1} BAP

Table 6 Comparison of means according to the effect of variety and type of explant on the regeneration of five wheat varieties. The data are presented by means (±SE)

Type of explant	Variety					Average
	Nassim	Rajae	Tigre	Wafia	Wissam	
ME	50.67 ±2.67^{cde}	68.00 ±3.27^{ab}	36.00 ±2.14^{e}	66.67 ±2.52^{abc}	48.00 ±2.62^{de}	53.87 ±1.82^{b}
MES	73.33 ±4.22^{ab}	80.00 ±3.90^{a}	44.00 ±3.49^{de}	80.00 ±4.36^{a}	58.67 ±5.68^{bcd}	67.20 ±2.51^{a}
Average		62.00 ±3.23^{b}	74.00 ±2.74^{a}	40.00 ±2.14^{c}	73.33 ±2.77^{a}	53.33 ±3.23^{b}

values (mean ±SE) followed by different superscripts letters are significantly different at $p = 0.05$

whether, for mature embryos or endosperm-supported mature embryo used as explants.

In general, since a long time 2,4-D was and still the most frequently used auxin to induce somatic embryogenesis. This was previously cited by Raziuddin et al. (2010). Yet, the callogenesis rate may vary not only with different concentrations of 2,4-D applied but also, according to varieties tested (Aydin et al., 2011; Mehmood et al., 2013). Our findings reveal that all the five varieties tested had a significant callogenesis frequency that fluctuates according to the applied concentration of 2,4-D (2; 3; and 4 mg.l^{-1} 2,4-D). However, 2 mg.l^{-1} 2,4-D presents the most effective concentration for inducing an optimal rate of compact callus comparatively to 3 mg.l^{-1} and 4 mg.l^{-1} 2,4-D. Many investigators such as Parmar et al. (2012) and Saeed et al. (2014) tested 2,4-D at different levels, have concluded that 2 mg.l^{-1} 2,4-D was the most efficient concentration to induce callus with good quality.

As far as the formation of embryogenic callus and their growth are concerned, the formation of embryogenic callus was highly dependent on 2,4-D concentration. Consequently, as described in the results, 2 mg.l^{-1} 2,4-D seems to be more effective in producing embryogenic callus in contrast to 3 mg.l^{-1} and 4 mg.l^{-1} 2,4-D where most of the calli maintained to grow were non-embryogenic (watery, loose). Moreover, according to daily observations, embryogenic calli were able to proliferate rapidly whereas the non-embryogenic calli were slowly growing under the same conditions. The same characteristics and remarks were acknowledged and stated by Parmar et al. (2012), Saeed et al. (2014), and Hakam et al. (2015). A recent study by Malik et al. (2021) suggested that the presence of auxin (2,4-D) in the culture medium is required for the callogenesis induction from mature embryo explants. By applying higher concentrations of 2,4-D, a greater number of primary calluses can be induced. Yet, the probability

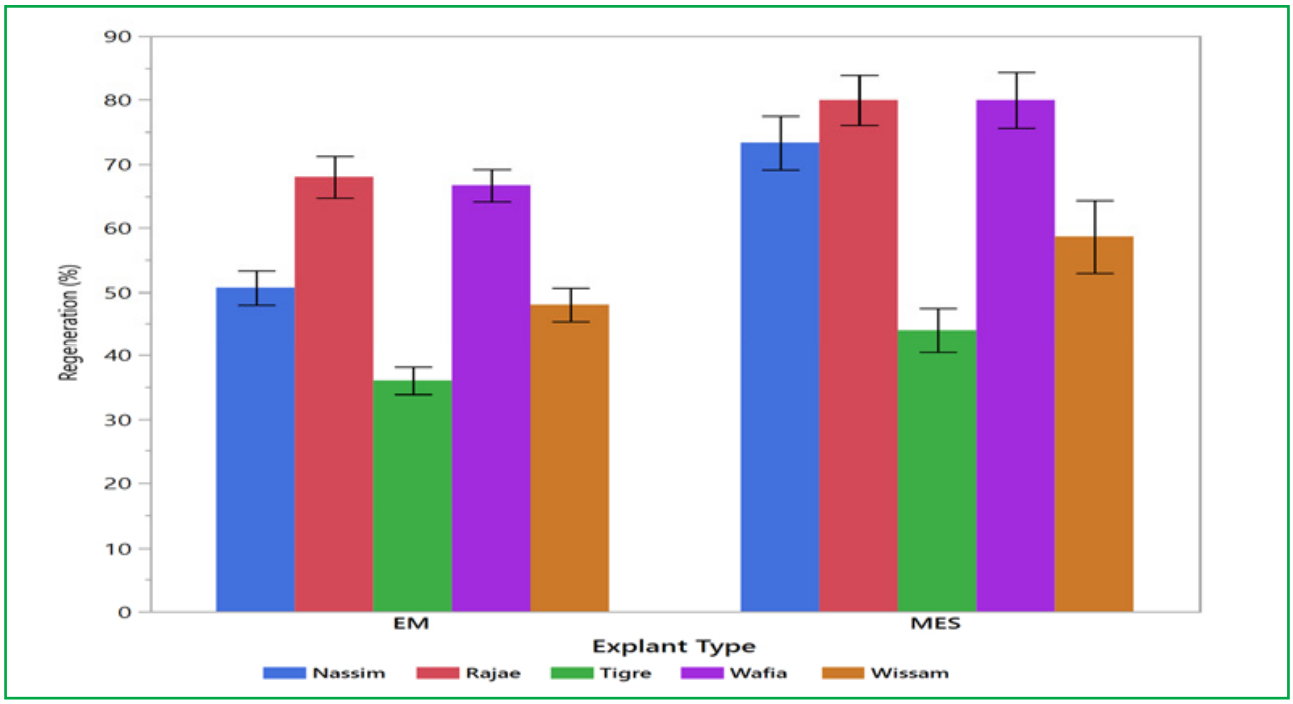

Figure 6 Effect of the explant type on regeneration of five varieties in wheat
data presented as mean (±SE)

of obtaining embryogenic embryos would be negatively influenced.

The use of the endosperm-supported mature embryo seems to be more competent to produce an interesting amount of vitro-plants regenerated from embryogenic callus compared to excised mature embryos. The results obtained by Aydin et al. (2011) corroborated ours. It was found that, the highest regeneration rate was recorded by MES. In addition, Mahmood et al. (2017) evaluated several types of explants including mature embryo, immature embryo, Endosperm-supported mature embryo, and shoot epical meristem. These authors reported that among the responses of the four explants used, the endosperm-supported mature embryo is the most suitable explant for plant regeneration.

Previously, Bartok and Sagi (1990) and Chen et al. (2006) originally questioned the better performance of mature embryos supported by the endosperm as explants. These authors proposed two reasons. The first may be due to the availability of more and readily available nutrition in the endosperm than in the artificial culture medium, while the second could be related to the presence of cells in the embryos that can receive signals from the endosperm and thus maintain a higher regeneration. Delporte et al. (2014) stated that EMs are now increasingly recognized as valuable explants for the development of regenerative cell lines in wheat biotechnology.

For regeneration, its frequency was significantly reliant to the age of incubated callus, and varieties tested. The same remarks were made by Haliloglu et al. (2005), who have explained this reliance as it is controlled by the genetic system in cereals. According to observations, 28 days old calli were efficaciously capable to regenerate shoots and roots at different percentages depending on the variety effect. In addition, the combination of auxin and cytokinin was highly recommended because morphogenic calluses can be enforced to regenerate shoots by increasing the concentration of cytokinin or decreasing auxin in the culture media (Mahmood et al., 2012).

In this manner, regeneration is a critical stage that is controlled by callus age, which is very important as a factor. Generally, calli with less age have more totipotency as compared to old calli (Rashid et al., 1994) originally quoted by Raja et al., 2009). In Fact, according to the same reseachers, the best age for regeneration is between 22 to 30 days old calli, although, callus with extended incubation period loses their regeneration ability due to repeated cell divisions, yet continue to form only roots. However, optimum callus age was also hormone and media-specific, as they signify importance that reinforces the regeneration capacity (Saad et al., 2004). Our results were in the same direction as Parmar et al. (2012), who revealed that MS Hormone-free medium is very favourable to initiate regeneration in callus induced originally at 2 mg.l^{-1} 2,4-D. Raja et al. (2009) mentioned also that, BAP is an important cytokinin in conferring competence to regenerate in cereals. Raziuddin et al. (2010) reported that combined to IAA at low levels BAP is taking an efficient role in shoot differentiation.

In the light of the obtained results, the use of the endosperm-supported mature embryo is highly recommended as this explant can provide a practical asset for *in vitro* culture in wheat, to compensate for the limited availability of immature embryos throughout the year, thus, facilitating the way for transformation studies of new genes in this plant.

The findings obtained in this study highlighted the key factors that mainly control the success of callogenesis and regeneration in bread wheat, namely the type of explant, the suitable concentration of 2,4-D, and the incubation duration. The protocol established in this work serves as a starting point since it enabled us to establish and set the *in vitro* culture conditions required to obtain a reliable regeneration system with the main goal of exploring the behaviour of these varieties and elucidating the coping mechanisms involved in salt stress conditions through *in vitro* selection.

4 Conclusions

An efficient regeneration system for bread wheat was established in this study. Significant rates of callus induction were recorded for all five varieties but vary with 2,4-D concentration. Additionally, embryogenic calli can be formed in all varieties, although in different percentages depending on 2,4-D concentration, which was found more effective at 2 mg.l^{-1} than at 3 and 4 mg.l^{-1}. As for the regeneration, regardless of the medium composition and the phytohormones applied (BAP and AIA), its frequency was highly dependent on the varieties tested. Concerning the explants, it has also an effect on regeneration. EMS were better than ME by presenting the highest percentage of regenerated plants overall in all varieties. Moreover, achieving significant induction of embryogenic callus with efficient regenerability for all five wheat varieties was promoted by employing the proper concentration of 2,4-D used at 2 mg.l^{-1} with an appropriate incubation period, the mature embryos supported by the endosperm as the explant type and the interaction between these factors. With this in mind, our results recommend using the endosperm-supported mature embryo (EMS) and the application of wounds around the embryo as a more effective method that

promotes the entrance of 2, 4-D, which in turn stimulates somatic embryogenesis, increases the ability of plants regeneration, and decreases the ability of embryos' early germination.

The success and triggering of any subsequent *in vitro* culture study, especially the selection of tolerant varieties, starts necessarily from a successful regeneration system that requires a better optimization of the factors controlling the culture (mineral and hormonal composition of the medium, type of explant, callus age).

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