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Somatic Embryogenesis for *Agropyron cristatum* on Murashige and Skoog Medium

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Abstract*—Agropyron cristatum* L. Gaertn. is a native grass of semiarid region in Iran which is quit resistant to cool and drought climate and withstand heavy grazing. This species has close phylogenetic relationship with *Triticum* and *Hordeum*. In this research, the effect of seven different concentrations of growth regulator 2,4-D on callus production and somatic embryogenesis of *A. cristatum* was investigated on Murashige and Skoog medium. The results showed that the rate of callus, embryo and neomorph were highest in 1 mg L^{-1} 2,4-D. Callus production was increased in 1 mg L^{-1} 2,4-D but dramatically decreased at 5.5 and 9 mg L^{-1} 2,4-D. The somatic embryos were observed at 1 and 4 mg L^{-1} 2,4-D but matured embryos and plantlet were only occurred at 1 mg L^{-1} 2,4-D. There were significant differences between 1 mg L^{-1} 2,4-D and other treatments for producing globular and torpedo embryos, plantlet, rooted callus and number of roots ($p<0.05$) and there was not any callus production and embryogenesis in control treatment without growth regulator.

***Keywords*—**2,4-D, callus production, somatic embryogenesis, *Agropyron cristatum*.

I. Introduction

MOST of the *Agropyron* spp. are originated from central Asia, including parts of Iran, Turkey, Afghanistan, Russia, and China [3]. These native wheatgrasses of semiarid regions in Iran are quite resistant to cool and drought climates and withstand heavy grazing, and thus are common on open and exposed knolls. The genus has a close phylogenetic relationship with *Triticum* and *Hordeum* genera and is widely used not only for livestock and wildlife feed, but also for preventing erosion and adding beauty to surroundings as ornamental grass in the parks, lawns and roadsides. For instance, although they are wild in nature, some of *Agropyron* spp. were planted extensively in arid rangelands of the United States and Canada [4]. However, despite their extensive use for cattle breeding and environmental protection, progress in production of improved cultivars by conventional approaches is still slow and inadequate. In this sense, the use of recently developed plant tissue culture methods (i.e., *in vitro* plant propagation through somatic embryogenesis or organogenesis) provides an effective alternative for producing novel germplasm and accelerating the development of new cultivars. Indeed, *in vitro* propagation methods enable fast multiplication of plants under controlled environmental conditions, production of clonal plants free of deseases, as well as medium- and long-term conservation of selected lines by means of slow growth storage and cryopreservation [8]. Somatic embryogenesis, in particular, has several advantages over plant regeneration by organogenesis; (i) single-cell origin avoids the emergence of chimeras in regenerated plants [9], (ii) increased regeneration rate is possible, even from stock cultures maintained for a long time [10], and (iii) the process allows specific changes that provide desirable, elite individuals through genetic engineering [1]. In this research, seeds collected from Golestan National Park of Iran were used as explant source, aiming to optimize reproducible protocols for morphogenic callus induction and somatic embryogenesis through seed-derived callus masses of *Agropyron cristatum* on liquid Murashige and Skoog (MS, Murashige and Skoog, 1962) media, independent of location-specific genotypes.

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II. Materials and Methods

Plant Material: The seeds of *A. cristatum* were collected from Golestan National Park (Iran), located at the east of Caspian sea, between 55° 43' to 56° 17' east and 37° 16' to 37° 31' north, with a mean annual precipitation of 300 mm and the average temperature of 12.7C°. Altitude of collections ranged from 1500 to 2000 m in hilly terrain of park. The habitats of *Agropyron* spp. were visited periodically (approximately in every 15 days) to record phenological stages. Spikes of vigorous plants were harvested at the seed ripening stage (i.e., during summer of 2008) to obtain viable seeds (Fig.1).

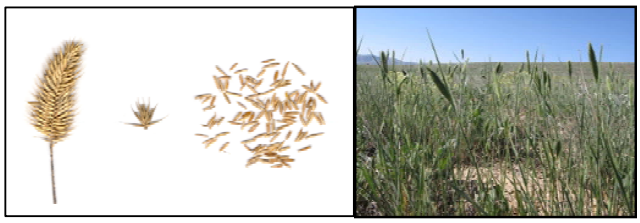

Fig. 1 Typical spike, spikelet and seeds of *A. cristatum* at Golestan National Park

Sterilization of explants: Mature embryos and caryopses of seeds were used as explants. Majority of seed bracts (lemma and palea) were detached from caryopses before sterilization. Fully matured, healthy and well dried seeds were surface sterilised by soaking in 70% ethanol for one minute and disinfected by 40 or 60% sodium hypochlorite (commercial bleach) solution for 20 min with constant shaking at 200 rpm.

The seeds were subsequently washed thoroughly (three to four times) with sterile distilled water until the trace of sodium hypochlorite was completely removed. Data were collected 30 days after being placed on medium, and was consisting of (i) decontamination rate (%), (ii) germination ability (%), and (iii) callus production (%).

Germination in vitro: A group of decontaminated seeds were germinated *in vitro* on liquid MS medium, either devoid of growth regulators (MS.0), or supplemented with 2,4-D (0.5, 1, 2, 4, 5.5, 9 mg/L). They were cultured at 26°C, under 16 hours photoperiod, provided by cool daylight fluorescent lamps (16 µmol m^{-2} s^{-1}). Data of germination rate was recorded daily after culture initiation.

Induction of callus masses and somatic embryogenesis: Seeds were cultured in liquid MS media. Semi-solid media were supplemented with 6 concentration of 2,4-D. Experiments were conducted under 16 h photoperiod, provided by cool daylight fluorescent lamps (16 µmol m^{-2} s^{-1}). Liquid MS media were supplemented with one of the following concentrations of 2,4-D; 0, 0.5, 1, 2, 4, 5.5 and 9 mg/L. The flasks were cultured under 16 h photoperiod, provided by cool daylight fluorescent lamps at 26ºC and 86 rpm with constant shaking. For somatic embryo induction, calluses were transferred to MS medium devoid of growth regulators (MS.0) after four weeks.

Somatic embryogenesis and plantlet regeneration from embryogenic callus: Plant regeneration was observed only when somatic embryos or embryogenic callus masses of *A. cristatum* were transferred to liquid MS.0 medium and cultured under continuous agitation. The results showed that the rate of callus and embryo for *A. cristatum* were higher when the liquid medium contained 1 mg/L 2,4-D (Fig. 2A). Callus production decreased dramatically when 2,4-D concentration was increased to 5.5 or 9 mg/L. This is consistent with earlier reports ([5], [6], [7]) where the use of high concentrations of 2,4-D for callus induction affected negatively the subsequent plant regeneration. As regards embryo maturation, it was possible to obtain somatic embryos in media containing 1 or 4 mg/L 2,4-D, however matured embryos and plantlets were observed only when the medium was supplemented with 1 mg/L 2,4-D (Fig. 2B-D). There were significant differences between the data obtained in medium containing 1 mg/L 2,4-D and other medium compositions as regards the production of globular and torpedo shaped embryos, plantlet regeneration, and rooted callus ($p<0.05$). On the other hand, callus production and embryogenesis were never observed on media devoid of growth regulator (Table 1 and 2).

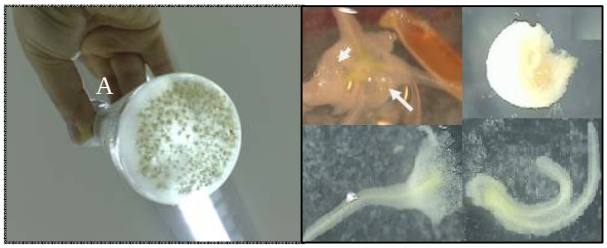

Fig. 2 Somatic embryogenesis of *A. cristatum* after subculture in MS media without 2,4-D A) embryogenic calluses, embryos and plantlets. B) Embryogenic callus, C) Globular embryo, D) Rooted plantlet, E) Neomorph

III. Conclusion

Effective indirect plant regeneration protocols for *in vitro* multiplication of *Agropyron* spp. are reported in the present study. It is hoped that results reported here will serve as beacon light for plant scientists to achieve a higher number of plants in the grass transgenesis. In conclusion, the protocol developed through this present investigation will be useful for large-scale regeneration of *Agropyron* spp. and possibly other related grass species used for semi steppe rangeland restoration. This work could also be helpful to researchers attempting to use biotechnological approaches to improve these grasses and provides a solid basis for the genetic transformation of these important monocot species. To our knowledge, there is no other report available in literature on *in vitro* plant regeneration of different *Agropyron* species in Iran.

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TABLE I
ANALYSIS VARIANCE OF DIFFERENT LEVELS OF EMBRYOGENESIS AND CALLUS PRODUCTION OF *A. cristatum* ACCORDING TO MEAN SQUARE IN LIQUID MS MEDIA WITH SEVERAL 2,4-D CONCENTRATIONS

Source	df	Globular embryos	rooted plantlet	Rooted callus	Callus without root	Root Number of callus
GR	6	0.791*	39.8*	114.1**	8.82**	2.404
Error	16	0.698	17.16	38.88	2.35	0.456

** Significant at 0.01 * Significant at 0.05

TABLE II
EFFECTS OF DIFFERENT 2,4-D CONCENTRATIONS ON EMBRYOGENESIS AND CALLUS PRODUCTION OF *A. cristatum* IN LIQUID MS MEDIA

2,4-D µm	Globular shape	Torpedo embryos	rooted plantlet	Rooted callus	Callus without root
0	0^{b}	0	0^{b}	0^{b}	0^{c}
2.26	0^{b}	0	0^{b}	0^{b}	2.75^{bc}
4.52	0.5^{b}	1	11.5^{a}	19.5^{a}	2.5^{bc}
9	0^{b}	0	0^{b}	0^{b}	3^{bc}
18	1.33^{a}	0	1.66^{b}	2^{b}	4.67^{ab}
25	0^{b}	0	0^{b}	0^{b}	1.25^{bc}
40	0^{b}	0	0^{b}	0^{b}	0.5^{c}

***** Column figures with the same letters were not significant