

Direct and indirect *in vitro* plant regeneration of two commercial cultivars of perennial ryegrass

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Key words: Abscisic acid (ABA), ‘Grassland’, *Lolium perenne* L., maltose, meristem tip, ‘Numan’.



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All relevant data are within the paper and its Supporting Information files.

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Abstract: Experiments were conducted on direct and indirect regeneration from the meristem tip and mature caryopsis explants of *Lolium perenne* L. ‘Numan’ and ‘Grassland’. De-husked caryopses were cultured both intact and longitudinally sliced on MS media supplemented with 2,4-D alone, and in combinations with BA. The highest percentage of callus induction obtained from intact-sliced caryopses were 71 and 87% for ‘Grassland’ on MS basal medium supplemented with 6 mg L⁻¹ 2,4-D + 0.02 mg L⁻¹ BA, and 5 mg L⁻¹ 2,4-D. While, for ‘Numan’, the highest callus induction was achieved by the same explants as 55% and 72% with 5 mg L⁻¹ 2,4-D + 0.02 mg L⁻¹ BA, and 4 mg L⁻¹ 2,4-D + 0.02 mg L⁻¹ BA, respectively. The best regeneration medium for ‘Grassland’ was MS medium supplemented with 10 g L⁻¹ maltose and 2 mg L⁻¹ ABA. In a separate experiment, meristem tip cultures were incubated on two type combination of plants growth regulators along with control treatment. The best regeneration rate was obtained in both cultivars on MS medium supplemented with 0.1 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ Kin. Plantlets with well-developed roots were transferred to greenhouse condition. Four weeks later, all acclimatized plants were survived.

1. Introduction

Turfgrasses control the soil erosion, carpet lawns, cover athletic fields, and beautify the environment. Perennial ryegrass (*Lolium perenne* L.) is one of the most important turfgrass species for sports fields, golf course fairways, as well as urban landscapes in the areas with temperate climate. That is the fast establishing component of lawn seed mixtures comprising slow growing species such as Kentucky bluegrass (*Poa pratensis* L.), and is used for winter overseeding on warm-season turfgrasses as well. However, the poor ability to survive in drought regions have limited its distribution. Therefore, improving drought tolerance is an important goal in perennial ryegrass breeding programs via classic or modern (genetic transformation) techniques. Callus production with good quality and efficient plant regeneration of various cultivars of perennial ryegrass is a requisite to the grass transformation techniques. The formation of embryo-

genic calli and plant regeneration were affected by several factors including genotype, explant tissue, culture medium and its supplements (Bhaskaran and Smith, 1990). Many studies have been done on improving tissue culture of perennial ryegrass. In perennial ryegrass, various explants including immature inflorescence, mature seed, leaf base, meristem tip and axillary bud have been used for callus induction, and direct and indirect plant regeneration (Dale, 1977; Dale and Dalton, 1983; Torello and Symington, 1984; Creemers-Molenaar *et al.*, 1989; Altpeter *et al.*, 2000; Bradley *et al.*, 2001; Can *et al.*, 2004; Salehi and Khosh-Khui, 2005; Newell and Gray, 2005; Altpeter, 2006). Among these explants, mature seeds are most commonly used for callus induction and related subsequent studies.

Perennial ryegrass is an outcrossing, wind-pollinated and highly self-incompatible species with abundant genetic variation in each cultivar (Mohr *et al.*, 1998; Smith *et al.*, 2001; Wang *et al.*, 2003; Bolaric *et al.*, 2005). Replacing the seed explants with other vegetative explants would omit this variation. *In vitro* meristem tip culture is an efficient method for obtaining virus free and identical to mother plant materials. In addition, meristem tip culture is an appropriate method for genetic transformation. In this method, the exposed meristem could directly be used for transformation; then after, will multiplied *in vitro*, or can form multiple shoot clumps that can be bombarded with the gene(s) of interest, and then each meristem will regenerated the mature plants. Therefore, development of an optimized and short time tissue culture protocol plays a critical role in successfully transformation of the grass species. To the best of our knowledge, there is no report on meristem tip culture of 'Grassland' and 'Numan' cultivars of perennial ryegrass.

In addition to, it has been shown stimulating effect of ABA on somatic embryogenesis of some grass species calli such as wheat (Qureshi *et al.*, 1989), maize (Close and Ludeman, 1987), Kentucky bluegrass (Van Ark *et al.*, 1991) and bermudagrass (Li and Qu, 2002).

The objectives of the present investigation were comparison of the callus induction on intact and longitudinally sliced caryopses, and evaluation of the effects of ABA and maltose combinations on plant regeneration of two *Lolium* sp. cultivars to optimize their tissue culture condition. Furthermore, a separate experiment was conducted to find a direct regeneration and fast tissue culture method for these cultivars.

2. Materials and Methods

Seed surface sterilization

Seeds of the two commercial cultivars of perennial ryegrass, 'Grassland' and 'Numan', were purchased from Iran Bazr Seed Company (Karaj, Iran). Seeds were soaked in 50% H₂SO₄ for 15-20 min to remove the husks (Salehi and Khosh-Khui, 2003). The de-husked seeds were surface sterilized with 70% ethanol for 1 min and immediately were treated with a house-hold bleach (5.25% available chlorine) for 20 min, then rinsed 6 times with sterile distilled water.

Callus induction

The surface sterilized seeds were used both intact and longitudinally sliced. For callus induction, MS basal medium (Murashige and Skoog, 1962) plus 3% sucrose supplemented with 4, 5, 6, 7, 8, 9 and 10 mg L⁻¹ 2,4-D alone and with 0.02 mg L⁻¹ BA. Intact and longitudinally sliced seeds were cultured on 80 mm petri dishes containing callus induction media.

After 4 weeks, the calli were separated, transferred to the same fresh media and maintained for 3 weeks. Then, the calli were transferred to optimum MS media, supplemented with 4 mg L⁻¹ 2,4-D + 0.02 mg L⁻¹ BA and 5 mg L⁻¹ for 'Numan' and 'Grassland', respectively. The number of seeds that induced callus was recorded according to seed viability. The callus induction rate was calculated as the number of caryopses which induced callus over the total number of explants plated × 100. All cultures were kept at 24±1°C in dark.

Plant regeneration

To determine the best regeneration media for 'Numan' and 'Grassland', the embryogenic calli were transferred to MS media containing 3% sucrose and 0, 10, 15 and 20 g L⁻¹ maltose with 0, 1.7, 2 and 2.3 mg L⁻¹ ABA. Filter-sterilized ABA was added after autoclaving when the media temperature dropped to 50°C.

Plant regeneration was scored 7-10 days after transferring the calli to the regeneration medium. The criterion used to determine regeneration was the formation of a distinguishable shoot at least 1 cm in length. Plant regeneration rate was defined as the percentage of callus that had regenerated shoots. The regeneration cultures were maintained at 24±1°C with a 16 h light/8 h dark cycle under a light intensity of 20 μmol m⁻² s⁻¹.

Meristem tip culture

In a second experiment, surface sterilized intact caryopses were incubated on 80 mm diameter petri dishes containing MS media, 3% sucrose, solidified

with 0.8% plant agar and were kept at $24\pm 1^\circ\text{C}$ in dark.

After emerging radicles, the petri dishes were transferred to 16h/8h light/dark photoperiodic condition. Meristem tips of 7-10 days old seedlings were excised by a Stereo microscope (C121506 Zeiss Stemi 1000 Binocular Stereo Zoom 0.7-3.5x Microscope 10x Eyepieces, Germany) and cultured on three different MS media including hormone-free MS, MS supplemented with 0.01 mg L^{-1} 2,4-D + 0.2 mg L^{-1} Kinetin, MS supplemented with 0.1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kinetin. All media were supplemented with 3% sucrose and pH was adjusted to 5.8 prior to autoclaving. The length of the meristem tips cultured ranged from 0.1-1 mm and consisted of the meristem dome plus one or several leaf primordia. After 2 weeks, the number of direct regenerated plantlets was recorded in each treatment based on green and healthy plantlets produced and then the percentage of direct regeneration of meristem tips was calculated. One hundred meristem tips were cultured in each petri dish. All media were placed in dark for 3 days at $24\pm 1^\circ\text{C}$ and then were transferred to 16h/8h light/dark photoperiodic condition. Light intensity was provided by cool-white fluorescent lamps at a photon flux density of $20\ \mu\text{mol m}^{-2}\text{ s}^{-1}$.

Statistical analysis

Complete randomized design with factorial arrangements was used for all experiments. For callus induction, each treatment had four replications, each replicate consisted of 100 seed. Experiments on direct and indirect regeneration were done with three replications. All data were analyzed with SAS

9.1 software and means were compared using LSD test at 5% level.

3. Results and Discussion

Effects of cultivars, explants and hormonal treatments and their interaction on callus induction

Plant growth regulators (PGRs) are specific molecules used in plants and supplemented at relatively low concentrations to work as signaling compounds for plant growth and development (Sauer *et al.*, 2013). The most extensively used and studied class of PGRs in plant tissue cultures are auxin and cytokinin (Ikeuchi *et al.*, 2013). Among auxin sources, 2,4-D is known as the most effective PGR to induce callus formation with stimulating cell elongation and enlargement in many plant species especially turfgrasses. In addition, low levels of cytokinins such as BA and BAP enhanced callus regeneration ability in several grass species (Zhong *et al.*, 1991; Van der Valk *et al.*, 1995; Chaudhury and Qu, 2000; Bradley *et al.*, 2001).

Calli were observed in 7 to 15 days after placing the explants on callus induction media. Their morphological appearance was hyperhydrated and white. After 4 weeks, three types of calli were observed including: i) white and hyperhydrated; ii) yellowish and friable; iii) compact, nodular, yellowish to opaque (Fig. 1 B-D). This variability is probably due to different tissues origin. Results showed that callus induction was notably increased by 28% in sliced caryopses (61.37) compared to intact caryopses (48.04). In addition, there was a significant difference

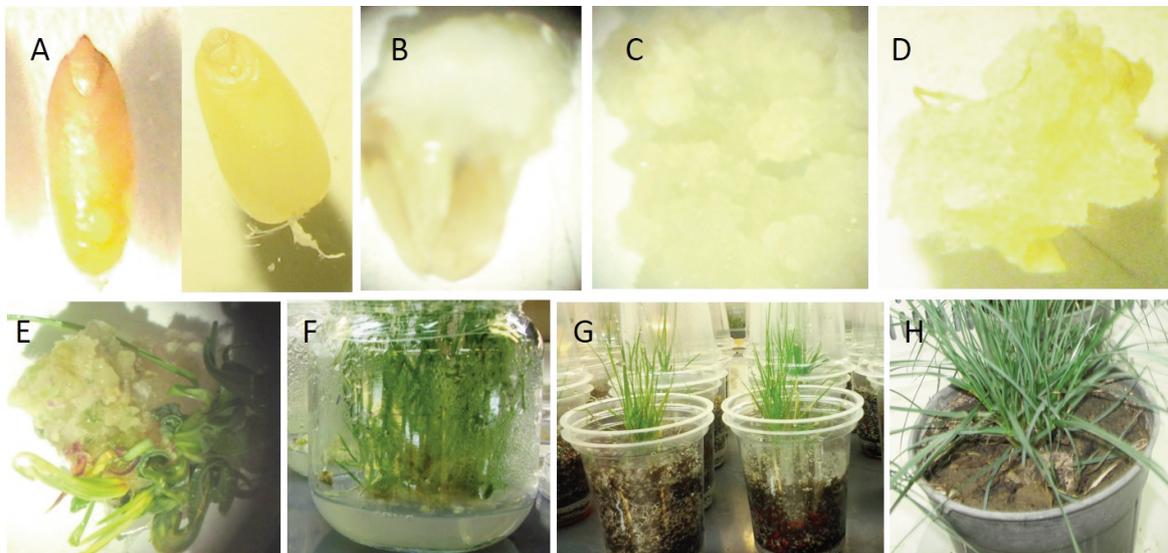


Fig. 1 - Steps of caryopses culture of two perennial ryegrass cultivars. A) The de-husked caryopses and surface sterilization of caryopses, right 'Grassland', left 'Numan'. B) Calli derived from longitudinally sliced caryopsis tissue, C) Embryogenic callus type ii. D) Embryogenic callus type iii. E) Green shoots produced on embryogenic callus. F) Shoot proliferation on the regeneration medium after two weeks. G) Plants acclimatized in soil mixture after one week. H) Healthy and green plants maintained in greenhouse after one month.

between two cultivars. The callus induction raised by 24% in ‘Grassland’ (60.57) compared to ‘Numan’ (48.83) (Data not shown).

In ‘Grassland’, the highest good quality callus induction was obtained at 5 mg L⁻¹ 2,4-D in sliced caryopses, while ‘Numan’ had the highest callus induction at 4 mg L⁻¹ 2,4-D with 0.02 mg L⁻¹ BA treatment in sliced caryopses (Table 1).

In ‘Grassland’, higher concentrations of 2,4-D declined callus induction with explants sliced and entire caryopses. However, more calli showed white color with increasing 2,4-D concentration in mentioned media. The effectiveness of 2,4-D in inducing the formation of callus is attributed to its main characteristic which can stimulate cell division of plant tissues and strongly suppress their organogenesis.

In a similar study, Liu *et al.* (2006) used 2,4-D and BAP for callus induction in perennial ryegrass. They found that the highest callus induction rate with the best callus quality was obtained on MS medium containing 5 mg L⁻¹ 2,4-D and 0.05 mg L⁻¹ BAP. Preliminary studies on the effectiveness of BAP on somatic embryogenesis in callus culture medium in several grass species were reported (Zhong *et al.*, 1991; Chaudhury and Qu, 2000; Bradley *et al.*, 2001). According to previous reports, the optimal BAP concentration in the induction medium should be 0.02-0.05 mg L⁻¹ for perennial ryegrass.

In this experiment, no significant difference was found on media supplemented with 0.0 or 0.02 mg L⁻¹ BA. However, this amount of BA had positive effect on morphology and cellular structure of calli

Table 1 - Interaction of different concentrations of 2,4-D and BA on callus induction of intact and sliced caryopses in two cultivars of *Lolium perenne* L.

2,4-D (mg L ⁻¹)	BA (mg L ⁻¹)	‘Grassland’		‘Numan’	
		Intact seeds	Sliced seeds	Intact seeds	Sliced seeds
4	0	49±3.9 de	69±6.0 bc	49±2.4 de	54±3.5d
	0.02	59±5.2 c	73±2.0 ab	55±1.8 d	41±2.9 e
6	0	51±3.4 de	77±3.8 a	30±2.9 g	62±3.6 c
	0.02	62±5.4 c	71±2.3 b	53±6.7 d	54±4.7 d
7	0	71±0.6 ab	60±4.8 c	41±3.2 e	58±1.7 cd
	0.02	55±3.5 d	75±5.5 ab	53±2.7 d	65±4.9 bc
8	0	53±1.1 d	73±6.3 ab	54±1.2 d	67±3.5 b
	0.02	46±5.0 e	69±3.4 bc	45±6.6 e	53±1.7 d
9	0	46±5.0 e	53±6.4 d	35±1.6 f	38±1.9 f
	0.02	38±6.6 f	57±2.5 c	29±2.4 g	53±1.9 de
10	0	43±7.9 e	54±4.1 de	38±2.8 f	41±3.2 e
	0.02	54±7.4 d	56±4.1 d	34±4.9 f	54±2.5 d

Values represent mean ± SE. Means with the same letter are not significantly different at p ≤ 0.05.

and their capacity to regenerate plantlets.

As previously stated, faster and more callus induction of longitudinally sliced caryopses in comparison with intact caryopses were detected. Increase in contact surface of tissue could be an important reason for callus induction. These findings are similar to other studies which have been conducted by Altpeter *et al.* (2000), Altpeter and Xu (2000), Bai and Qu (2001) and Bradley *et al.* (2001) on *Lolium* sp. and *Festuca* sp. Bai and Qu (2001) improved both callus production and regeneration rate of tall fescue, *Festuca arundinacea* Schreb. with slicing seeds in callus culture medium. Also, similar inclusion on chopping the mature seeds of *Lolium* sp. and *Festuca* sp. to suppress germination and stimulation of callus production were reported (Altpeter *et al.*, 2000; Altpeter and Xu, 2000). Although, Salehi and Khosh-Khui (2005) obtained contrary results by using horizontally sliced caryopses of *Lolium* sp. cultured on callus induction medium. These results might be related to genotypic differences and callus origin. These results might be used as a general application for other grass species.

Interaction effect of ABA and maltose on plant regeneration

The highest regeneration rates were recorded for both cultivars on MS medium supplemented with 30 g L⁻¹ sucrose, 10 g L⁻¹ maltose and 2 mg L⁻¹ ABA. As shown in figure 2, ‘Grassland’ has higher regeneration rate (~13%) than ‘Numan’. Incidentally, maltose had not notably effect on plant regeneration percentage in both cultivars lonely but ABA concentrations showed significant effects on plant regeneration rate (Fig. 2). This result can be referred to the role of ABA in maturation of embryos (Finkelstein *et al.*, 1985).

The most important source of energy for plant growth in the culture medium is carbon. Several

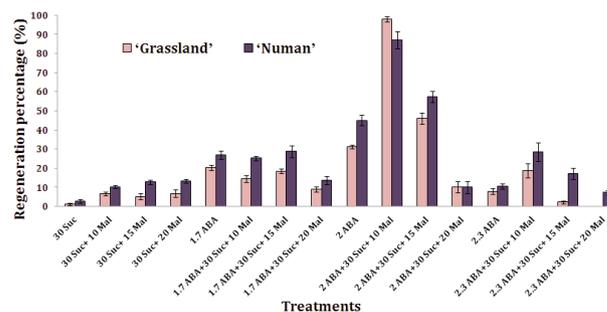


Fig. 2 - Regeneration rates of two cultivars of *Lolium perenne* L. in combination concentrations of ABA (0, 1.7, 2 and 2.3 mg L⁻¹), and maltose (0, 10, 15 and 20 g L⁻¹ Mal) under same concentration of sucrose (30 g L⁻¹ Suc).

studies have reported the effects of carbohydrates such as sucrose and maltose on different plant species like wild cherry, rubber tree, oilseed rape and sugarcane (Reidiboym-Talleux *et al.*, 1998; Blanc *et al.*, 1999; Slesak and Przywara 2003; Gill *et al.*, 2004). Maltose is the best carbon source for callus induction and regeneration medium. Furthermore, maltose, as an osmoticum, plays a major role in the optimization of callus cellular environment and protect calli by reducing ethylene (Darachai *et al.*, 2004; Zaidi *et al.*, 2006).

The regeneration rate was affected by using maltose, to almost five-fold increase at 20 g L⁻¹ maltose compared to 30 g L⁻¹ sucrose in both cultivars studied (Fig. 2). In addition, ABA increased the regeneration rate ~25 and 16 fold at 2 mg L⁻¹ in comparison with 0 mg L⁻¹ in ‘Grassland’ and ‘Numan’, respectively.

The combination of maltose and sucrose and ABA at high concentrations had no promoting effect on plant regeneration in both cultivars. In ‘Grassland’, significant declines was observed at 2.3 mg L⁻¹ ABA + 20 g L⁻¹ maltose (71.3%) and 1.7 mg L⁻¹ ABA + 10 g L⁻¹ maltose (100%). According to the results, both cultivars showed different responses at high concentrations of maltose and ABA combination. Generally, the regeneration rate dramatically reduced by 100% at 2.3 mg L⁻¹ ABA + 20 g L⁻¹ maltose in ‘Numan’ compared to ‘Grassland’ (Fig. 2).

A probable reason for reducing the regeneration percentage is related to sucrose in regeneration media that stimulates the ethylene production in plant tissues which can cause browning of calli. On the contrary, maltose might protect the calli from browning. However, beneficial effect of maltose was observed on embryogenesis and regeneration of cereals such as rice, wheat and perennial ryegrass, but the mechanisms of maltose role in tissue and cell culture media is not yet completely known.

There were significant differences between two cultivars according to plant regeneration rates. Our results are in accordance with previous studies on

Kentucky bluegrass (Van Ark *et al.*, 1991), Zoysiagrass (Dhandapani *et al.*, 2008), and bermudagrass (Li and Qu, 2002). According to the results of Van Ark *et al.* (1991), by adding ABA to regeneration medium, the percentage of calli with somatic embryos or embryo-like structures increased (up to 29.6%) as compared to the control (16.4%). In addition, Dhandapani *et al.* (2008) found that ABA significantly increased embryogenic callus formation from stem nodes, but not from young inflorescences. ABA not only promotes the transition of somatic embryos from the proliferation to the maturation phase (Langhansova *et al.*, 2004), but it also enhances embryo quality by increasing desiccation tolerance and preventing precocious germination (Li *et al.*, 1997; Robichaud *et al.*, 2004; Vahdati *et al.*, 2006; Rai *et al.*, 2008). Ultimately, based on mentioned authors results, the role of ABA on increasing somatic embryogenesis and regeneration were affected by several factors including origin of explant, physiological status and environmental condition.

Direct regeneration in meristem tip culture

An efficient and rapid method was developed for direct regeneration of two cultivars of perennial ryegrass. The results of meristem tip culture provided good information regarding changings in PGRs and their effects on plantlets growth and development. There was significant difference between two cultivars in plantlet regeneration rate. In ‘Grassland’, the overall regeneration rate of meristem tips was about 63.5%, while this parameter was approximately 55.7% in ‘Numan’.

Meristem elongation and development as a single plantlet on MS medium without PGRs was observed (Fig. 3C). More plantlets were obtained from ‘Numan’ meristem tips in comparison to ‘Grassland’ on hormone-free MS medium (Fig. 3D). The same results were previously reported on *Curculigo latifolia* (Babaei *et al.*, 2014). In the present study, overall regeneration percentage of both cultivars on MS media was obtained approximately 41%, while in

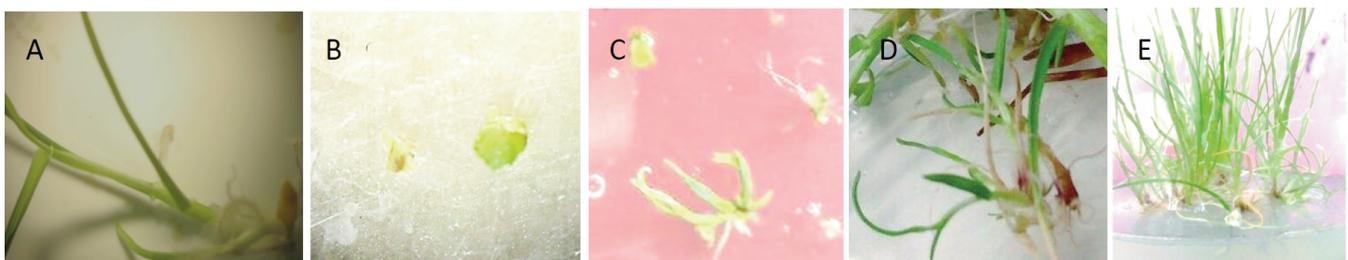


Fig. 3 - Steps of meristem tip culture. A) Ten days old seedling. B) Isolated meristem tips. C) Meristem tip cultured on MS medium. D) Meristem tips cultured on MS medium supplemented with 0.01 mg L⁻¹ 2,4-D + 0.2 mg L⁻¹ Kin. E) Meristem tips on MS medium supplemented with 0.1 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ Kin.

Dale (1977) investigation, overall regeneration percentage of nine species of grasses was reported about 11% on hormone free MS medium.

By adding plant growth regulators to the media, more plantlets and tillers grew in both cultivars. As shown in Table 2, an increased regeneration percentage of 35% and 37% by adding 0.01 mg L⁻¹ 2,4-D + 0.2 mg L⁻¹ Kin and 53% and 48% by increasing Kin from 0.2 to 0.5 mg L⁻¹ was observed in ‘Grassland’ and ‘Numan’, respectively. These results show that an exogenous supply of growth regulators is required or can be beneficial for the regeneration of whole plants from stem apices as stated in several previous studies (Dale, 1977).

Table 2 - The regeneration percentage from meristem culture of two cultivars of perennial ryegrass on MS hormone-free and with PGRs media

Medium	‘Grassland’	‘Numan’
MS	43±3.3 de	38±1.15 e
0.01 2, 4-D + 0.2 Kin	58±1.15 c	52±3.06 c
0.1 2, 4-D + 0.5 Kin	89±2.40 a	77±3.71 b

Values represent mean ± SE. Means with the same letter are not significantly different at p ≤ 0.05.

In his first study on meristem tip culture of *Lolium* species, Dale (1977) stated that the best regeneration rate was obtained on MS medium compared to the other media. The higher regeneration rate was obtained for *L. multiflorum* (92%) on the medium containing 0.01 mg L⁻¹ 2,4-D + 0.2 mg L⁻¹ Kin and for *L. perenne* (54%) on medium containing 0.5 mg L⁻¹ 2,4-D + 0.02 mg L⁻¹ Kin. According to our results, the size of the meristem tips cultured usually affects their response in culture. In ‘Numan’ large meristem tips survived at a higher rate than small ones and generally grew more rapidly. As shown in figure 4, there was a positive relation between survival rate of meristem and meristem size in both cultivar. Differences in survival rate between cultivars also seemed to be related to the meristem tip size. This is in accordance with previous results reported by Dale (1977) in four genera of grasses including: *Lolium*, *Festuca*, *Phleum* and *Dactylis*.

4. Conclusions

In summary, for both cultivars, the best callus induction media with using longitudinally sliced caryopses at 5 mg L⁻¹ 2,4-D and 4 mg L⁻¹ 2,4-D + 0.02 mg

L⁻¹ BA was selected. In sliced caryopses more callus induction was observed in both cultivars. The best regeneration medium for them was also recommended. Generally, low concentrations of maltose and ABA was more effective on regeneration rate of two cultivars. In addition, an efficient and rapid procedure for direct *in vitro* regeneration of two perennial ryegrass cultivars has been established. Despite, MS hormone-free medium was an acceptable medium for regeneration of meristem tips but the highest regeneration rate of meristem tips was observed with 0.1 mg L⁻¹ 2, 4-D + 0.5 mg L⁻¹ Kin in both cultivars. There was a positive relation between length of meristem tips and their survival percentage. It is now possible to test the effectiveness of this technique to produce virus-free plants. Moreover, the results can definitely improve the transformation efficiency of these cultivars.

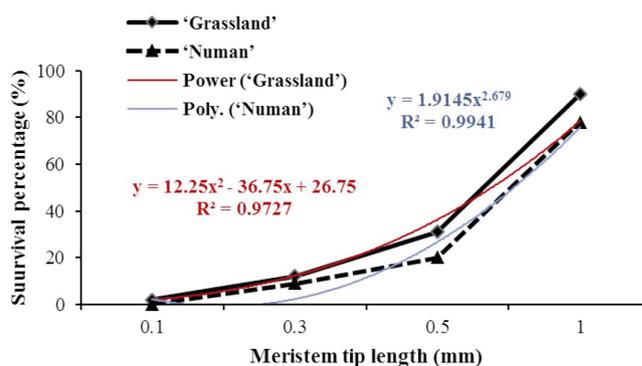


Fig. 4 - Survival percentage as a function of meristem tip length. The red and blue lines are the best fitness for ‘Grassland’ and ‘Numan’, respectively.

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