

Improvement of *In Vitro* Plant Regeneration for Genetic Transformation of Argentinean Onion Varieties

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ABSTRACT

Incorporation of certain genetic traits into plants depends on the existence of an appropriate transformation protocol for the species. The development of such a biotechnological approach to insert new attributes in onion (*Allium cepa* L.) did not exist until a few years ago. In spite of this achievement, studies aimed at the optimization of the transformation protocol adjusted for local conditions, including genotypes, are still needed. Indeed, genotypes are of extreme importance in the case of onion since they exhibit a wide variable *in vitro* behavior. On the other hand, the preference of both producers and consumers from Argentina and importer countries is on distinctive local cultivars. Thus, it is of utmost importance to study and adjust the conditions to establish a protocol leading to stable genetic transformation of local onion cultivars. The main constraint to achieving this goal is the establishment of an efficient system for *in vitro* plant regeneration.

Keywords: *Allium cepa*, callus, culture media, explants, growth regulators, transformation, transgenic

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, 6-(γ - γ -dimethylallyl-amino)-purine; NAA, α -naphthaleneacetic acid; BAP, 6-benzylamino-purine; BDS, basal salt and vitamins culture medium according to Dunstan and Short (1977); MS, basal salt and vitamins culture medium according to Murashige and Skoog (1962)

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INTRODUCTION

In 2004, the production of onion (*Allium cepa* L.) in Argentina reached 700,000 tons most of them belonging to local onions cultivars of the long days type Valenciana. Argentina is the main exporter of onion in Latin America with 83% of this vegetable going to Brazil and 7% to the European Union (Galmarini *et al.* 2003). Regarding the varietal availability, there are very good quality and highly productive materials, presenting excellent acceptance in the internal and external market, but they are reduced in number and susceptible to diseases (Galmarini *et al.* 2003). Conventional plant breeding has greatly contributed to provide cultivars still in cultivation; however, their potential to solve the main problems of the crop is limited. Besides, foreign cultivars showing good performance in our agroclimatic conditions do not exist.

In Argentina, just one cultivar, 'Valcatorce INTA', occupies more than 85% of the cultivated area in the southern region of Buenos Aires province, and it is also the main exported onion variety. The long day Valenciana-type cultivar 'Valcatorce INTA' is preferred by the producers and buyers because of its excellent traits, i.e., superb quality, high productivity, rusticity – mainly at post-harvest due to the thick and resistant cataphylls and a tight bulb neck – good resis-

tance to damage by mechanical shock, delayed sprouting, medium size, good shape and colour and well adapted to agroclimatic local conditions, storage, processing, packing and transportation (Galmarini 2000). Other outstanding cultivars are 'Valuno INTA', 'Cobrizo INTA', 'Grano de Oro', and 'Torrentina' which is an intermediate day's onion. However, all cultivars have some problems, mainly the susceptibility to diseases.

It is reasonable to argue that transgenesis would help to keep the good characteristics of some of the local cultivars (i.e. productivity, quality, commercial acceptance) while allowing the incorporation of desired traits (i.e. resistance to biotic and abiotic stress, resistance to herbicides, improvement of the nutritional quality), otherwise difficult to get through traditional breeding techniques (Eady 1995). During the breeding process, undesirable genome regions from the donor genotype are still present and must be removed from the acceptor genotype. This is of utmost importance in onion due to its biennial nature, the genetic heterogeneity of the cultivars and the difficulty in obtaining controlled crossings successfully (Dowker 1990; Pathak and Veere Gowda 1993). In contrast, linkage drag is avoided in genetic transformation.

Genetic transformation in onion was reported for a limited number of varieties (Eady *et al.* 2000; Zheng *et al.*

Table 1 Stable genetic transformation of onion (*Allium cepa* L. *cepa*) and shallot (*A. cepa* L. *aggregatum*).

Horticultural Group ^a	Cultivar/genotype	Target tissue	Transformation method	Expressed Gen	Reference
Onion	open-pollinated 'Canterbury Longkeeper'	immature zygotic embryos	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> and <i>gfp</i>	Eady <i>et al.</i> 2000
Onion	open-pollinated Onion 'Sturon' and 'Hyton'	calli from mature zygotic embryos	<i>A. tumefaciens</i>	<i>gusA</i> and <i>hpt</i>	Zheng <i>et al.</i> 2001
Shallot	open-pollinated Shallot 'Kuning'	calli from mature zygotic embryos	<i>A. tumefaciens</i>	<i>gusA</i> and <i>hpt</i>	Zheng <i>et al.</i> 2001
Onion	open-pollinated 'Canterbury Longkeeper'	immature zygotic embryos	<i>A. tumefaciens</i>	<i>bar</i> and antisense versions of <i>alliinase</i>	Eady <i>et al.</i> 2002, 2003a, 2003b
Shallot	open-pollinated 'Tropix' and 'Kuning'	calli from mature zygotic embryos	Microprojectile acceleration	<i>gusA</i> , <i>hpt</i> and <i>Bt</i>	Zheng <i>et al.</i> 2005
Onion	open-pollinated 'Canterbury Longkeeper'	immature zygotic embryos	<i>A. tumefaciens</i>	GFP and <i>nptII</i>	Eady <i>et al.</i> 2005
Onion	inbred line KU-31	calli from seed radicles	Microprojectile acceleration and <i>A. tumefaciens</i>	Positive selectable marker <i>pmi</i>	Aswath <i>et al.</i> 2006
Onion	open-pollinated HG400B	calli from stem discs	Microprojectile acceleration	OSISAP1 (<i>Oryza sativa</i> stress-associated protein gene)	Xu and Cui 2007

^a According to Hanelt (1990)

2001; Aswath *et al.* 2006) (Table 1). Researchers working in this field recognize a great difficulty in obtaining transgenic plants, and in the few successful cases, it occurred with very low efficiency, being limited by several factors, mainly a low *in vitro* regeneration capability (Eady *et al.* 1998; Zheng *et al.* 1998, 1999; Marinangeli *et al.* 2006). This capacity is mainly dependent on genotype, although optimized *in vitro* culture conditions are also important.

The performance of transgenic varieties is not only dependent of the appropriate expression of the specific attribute of the transgene, but also of the agronomic potential of the acceptor genotype. Thus, the transgene of interest should ideally be integrated into superior cultivars and advanced lines that could be introduced in breeding programs or immediately incorporated to cultivated onions (Lydiat *et al.* 1995; Kumar *et al.* 2005). Table 1 shows the low number of *Allium cepa* cultivars (onions and shallots) where transformation could be obtained.

Important progress has been made in the setting up of a protocol for genetic transformation of local onions in Argentina. This work was done mainly with 'Valcatorce INTA', the wide spread cultivar, with other cultivars of minor relevance such as 'Torrentina', 'Cobriza' and 'Grano de Oro', and also with other proven genotypes for the sake of comparison. In order to find potential target explants for transformation, explant types, cultural conditions and regeneration systems were evaluated (Marinangeli *et al.* 2005). To recover the transformation events, different selection markers and conditions were tested. A number of parameters that influence particle-mediated gene delivery by gene gun and *Agrobacterium tumefaciens*-mediated transformation were adjusted, leading to reliable transient expression. These parameters included helium pressure and target distance for the biolistic method, as well as bacterial strain, inoculation and co-cultivation conditions and explant type and pre-treatment, among others, for *A. tumefaciens*-mediated transformation. Finally, several transformation experiments were performed using a combination of the best conditions found, but it was not possible to regenerate transgenic plants. Arguments for this result and alternative substitute methodologies were proposed (Marinangeli *et al.* 2006).

With regard to transgenic products, the current perception constrains of consumers, especially from onion importer countries, would make almost impossible the international commercialization of transgenic onion cultivars. However, considering the rapid expansion of this technology in many food crops of economical value, the availability of this biotechnique for onion is important. Thus, it is necessary to advance in the knowledge of the gene transformation possibilities in onion as a mid and long term strategy for breeding of this species. Moreover, it should also be

considered that genetic transformation and expression provides a useful tool for basic studies.

The main purpose of this work was to study and adjust the conditions for the establishment of a protocol leading to a stable genetic transformation of local onion cultivars. In the following sections we report on the advances reached on *in vitro* culture procedures consistent with the above mentioned objective.

IN VITRO CULTURE OF ONION

The optimization of an *in vitro* plant regeneration system for a selected genotype is a pre-requisite in an efficient protocol for genetic transformation, either via tissue culture or isolated cells (Birch 1997). Hence, in order to develop an efficient protocol for genetic transformation of Argentinean onion cultivars, it is necessary to establish reliable *in vitro* culture systems in order to obtain target explants for transformation which lately would lead to regeneration of transgenic plants.

The *in vitro* culture of plant cells, tissues and organs has been widely used with propagation purposes and for production of plants free of pathogens in several cultivated species of the *Allium* genus. On the other hand, *in vitro* culture has also been a useful tool to increase the genetic variability and even to produce new cultivars (Novak 1990). Hence, these *in vitro* techniques are important due to their potential for onion breeding when providing an adequate way for the introduction of new traits which otherwise are difficult or impossible to be incorporated using traditional methods.

There are several methods available for the *in vitro* culture of species of the *Allium* genus. Some examples with special emphasis in *A. cepa* are the following:

a) Direct shoot multiplication from meristematic apex or axillary buds (Havel and Novak 1985; Ikeda and Imoto 1991; Kohmura *et al.* 1994); b) Embryo rescue in interspecific crossings and shoot proliferation (Nomura and Makara 1993; Nomura *et al.* 1994); c) Direct formation of adventitious shoots obtained from explants of the basal plate (Hussey and Falavigna 1980) or from immature floral organs (Matsubara and Hihara 1978; Pike and Yoo 1990); d) Indirect formation of adventitious shoots and/or somatic embryos via calli formation starting from root segments, basal plate, leaves, immature umbels (IU), ovules and zygotic embryos (MZE) (Dunstan and Short 1977; Van der Valk *et al.* 1992; Juntawong *et al.* 1993; Zheng *et al.* 1998); e) Plantlet regeneration starting from protoplasts in onion and other species of *Allium* (Hansen *et al.* 1995).

Methods a and c offer genetic stability of regenerated plants (clones), while method d can generate somaclonal variation, which is of interest in breeding programs when the natural variability of the species is low, as it is the case

of onion (Donovan *et al.* 1994). The early rescue of embryos is used to overcome post zygotic incompatibility problems in interspecific hybrids; thus, a significant increase in the recovery of hybrid plants obtained from crosses among species of *Allium* is allowed (Nomura *et al.* 1994).

Usually, *in vitro* culture in onion has been done by direct organogenesis, i.e. promoting bulblet development (Mohamed-Yasseen and Splittstoesser 1992; Keller 1993) or by shoot formation from the explant, avoiding the callus stage (Hussey and Falavigna 1980; Pike and Yoo 1990; Kahane *et al.* 1992a). Microplants have also been obtained by indirect organogenesis (Van der Valk *et al.* 1992; Mohamed Yasseen and Splittstoesser 1992; Juntawong *et al.* 1993; Zheng *et al.* 1998). Somatic embryogenesis has been obtained from callus of *A. cepa* (Hussey and Falavigna 1980; Pike and Yoo 1990; Kahane *et al.* 1992a) and haploid plants were induced from *in vitro* culture of non-pollinated ovules (gynogenesis) (Campion and Alloni 1990); haploid plants were also produced using immature floral buds (Bohanec *et al.* 1995).

In vitro tissue culture of onion has been started from a variety of organs and explants, some of which are: seeds (Mohamed Yasseen and Splittstoesser 1992; Keller 1993), base of the young inflorescences (Matsubara and Hihara 1978), bulb sections (Dunstan and Short 1977; Hussey and Falavigna 1980), immature floral buds (Pike and Yoo 1990; Martínez *et al.* 2000; Ponce *et al.* 2006), non-fecundated ovules (Campion and Alloni 1990), basal plate (Kahane *et al.* 1992a; Juntawong *et al.* 1993), MZE (Van der Valk *et al.* 1992) and root tips (Quintana Sierra *et al.* 2005).

Different responses have been obtained depending on the type of starting explant. They involve either direct or indirect organogenesis or even somatic embryogenesis. In turn, those different pathways influence the *in vitro* plant regeneration potential (Novak *et al.* 1986), and, of course, these responses are dependent on genotype (Zheng *et al.* 1998), composition of the culture media (mainly plant growth regulator (PGR) composition) and the environmental conditions. In onion it the red/far-red ratio of light irradiation and the photoperiod type is also relevant (Kahane *et al.* 1992b).

A wide variety of explants (e.g., roots, leaves, floral parts) of *Allium* species can be aseptically cultivated in nutrient media supplemented with PGRs to produce calli with non differentiated tissues. If these calli are friable they can be disaggregated and transferred to a liquid nutrient medium to obtain a cell suspension culture. These cell suspensions consist on cellular aggregates and isolated cells. Nevertheless, the *Allium* species have a strong tendency to form firm and compact calli, and therefore, the establishment of cell suspensions has not been well developed (Novak 1990).

In *Allium* genus, plantlet regeneration was achieved starting from cell suspensions in the following species: *A. cepa* (Hansen *et al.* 1995), *A. fistulosum* (Song and Peffley 1994), *A. fistulosum* × *A. cepa* (Song and Peffley 1994), *A. sativum* (Barrueto Cid *et al.* 1994, Fellner and Havranek 1994), *A. longicuspis* (Fellner and Havranek 1994), and *A. ampeloprasum* (*A. porrum*) (Buitveld 1994; Schum *et al.* 1994).

Calli induction and establishment of cell suspensions, their multiplication and further plantlet development are processes dependent on different factors (Novak 1990), being the most important: the genotype of the donor plants, their growth environment, the type of explant, the combination and concentration of PGRs present in the culture media, and the environmental conditions settled for *in vitro* culture.

As target for genetic transformation, the intact plant can be used as well as organs, tissues, cells and protoplasts. Target explants can also be generated *in vitro*, e.g. somatic embryos and calli (Birch 1997; Hansen and Wright 1999).

In *Arabidopsis thaliana*, *in planta* transformation has been widely used with research purposes because of its simplicity and efficiency. The use of this technique has been a key point for building the complete genome map for this

species (Bent 2000). *In planta* transformation is not possible in many plant species, mainly due to the difficulty of obtaining a tissue and/or organ able to differentiate high quantity of propagules *in vivo*, which in turn would be adequate targets for the implementation of some of the usual methods for transformation (Bent 2000). Onion plantlets were obtained *in vivo* at high frequency from immature reproductive organs (Brewster 1994).

Direct regeneration of transgenic plants from organs used as target explants have also been used in protocols of several species (Christou 1997). Direct regeneration of multiple shoots has been reported from several organs of onion plants, as it was previously mentioned, thus presuming the possibility of obtaining transgenic plants.

In transformation protocols of several species, calli induction and plantlet regeneration or direct embryogenesis are frequent strategies (Hansen and Wright 1999). The most successful regeneration systems suitable for onion transformation protocols use immature zygotic embryos (IZE) and mature zygotic embryos (MZE) as starting materials and also target tissues consisting of actively dividing cells, i.e. IZE (Eady *et al.* 1998, 2000) or embryogenic calli (Zheng *et al.* 1998, 1999, 2001) (Table 1).

For local cultivars, and especially for 'Valcatorce INTA', have been developed different ways of obtaining plantlets starting from explants, which could be targets for genetic transformation, thus increasing the probabilities of obtaining transgenic plants.

Direct organogenesis in local onion 'Valcatorce INTA'

Onion plants regeneration through the callus pathway is variable and inefficient and also strongly dependent on the genotype (Phillips and Luteyn 1983). In some recalcitrant species it is possible to obtain transgenic plants starting from apical meristems followed by shoot multi-sprouting (Christou 1997). As previously mentioned, an appropriate *in vitro* culture protocol for regeneration of onion plants is a pre-requisite for the subsequent application of other plant biotechniques. Several possible regeneration protocols were reported for species of the *Allium* genus (Novak 1990) with variable success, examples are the microshoot multiplication from axillary or adventitious buds (Novak *et al.* 1986; Kahane *et al.* 1992a) and the plantlet regeneration starting from floral organs in different stages of development, e.g. inflorescences (Matsubara and Hihara 1978), and flowers (Matsubara and Hihara 1978; Pike and Yoo 1990).

Working with the 'Valcatorce INTA' cultivar, multiple shoot proliferation was obtained from splitted plantlets produced *in vitro* from MZE extracted aseptically from seeds (Marinangeli *et al.* 2005). The seeds were initially subjected to double disinfection (Marinangeli *et al.* 2005) and the embryos were extracted according to the technique described by Van der Valk *et al.* (1992). Then, they were cultivated in BDS medium (Dunstan and Short 1977) with different NAA (α -naphthalenacetic acid) and BAP (6-benzylamino-purine) concentrations. At the time of the first subculture, simple plantlets were obtained in all treatments and by the second one; plantlets were divided into halves to break the apical dominance and to stimulate the sprouting of axillary and adventitious buds. During the two following sub-cultures increasing amounts of plant propagules were obtained by increasing the content of NAA and BAP in the nutrient medium, reaching a maximum of 8.3 shoots per explant with 1 mg.L⁻¹ NAA and 10 mg.L⁻¹ BAP and 68% of the explants with multiple sprouting. This multiplication ratio is comparable to the reported using other onion varieties but with lower content of NAA and an equivalent content of BAP (Kahane *et al.* 1992a).

The reproductive meristem in early stages of floral differentiation could provide a juvenile tissue able to differentiate shoots from one or few cells, thus providing a potential target for genetic transformation. This phenomenon has been observed spontaneously *in vivo*, as a response to high

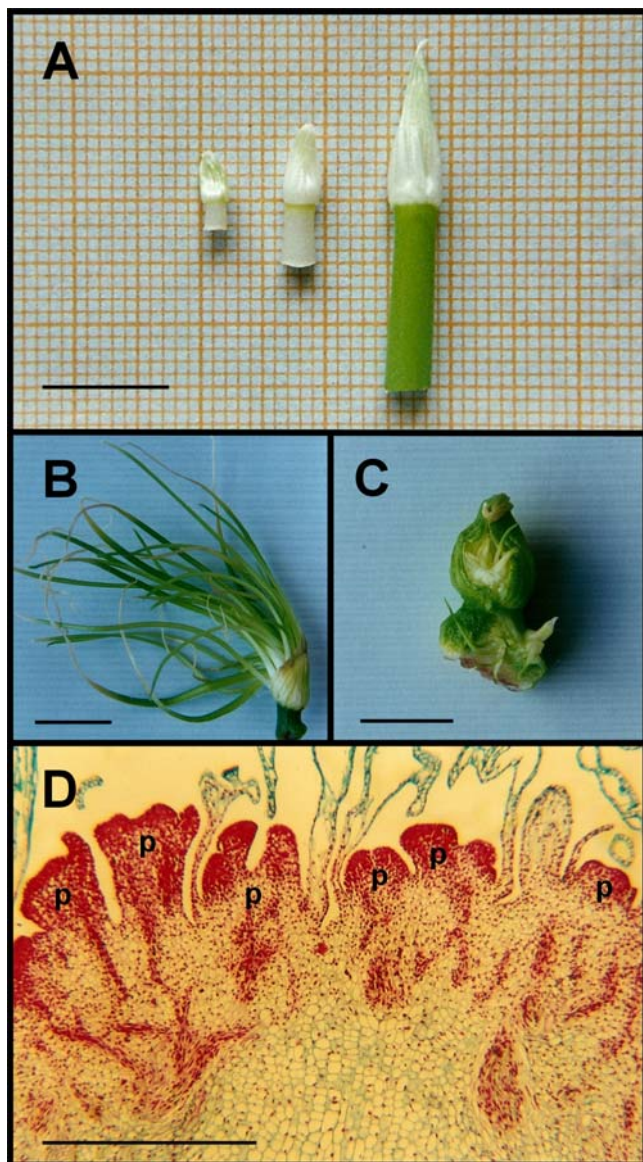


Fig. 1 Direct shoot proliferation from immature umbels (IU). (A) IU used as explants classified in three sizes according their diameter at the base: 1.2 - 2 mm, 2 - 4 mm, and 4 - 6 mm. (B) IU after 35 days in BDS medium with 0.01 mg.L⁻¹ NAA and 1 mg.L⁻¹ BAP. (C) IU after 35 days in BDS medium with 0.01 mg.L⁻¹ NAA and 10 mg.L⁻¹ BAP. (D) Histological section of a 2-4 mm IU cultivated during 11 days in BDS medium with 0.01 mg.L⁻¹ NAA and 1 mg.L⁻¹ BAP. It can be seen shoot primordia (p). Scale: bars represent 1 cm in A, B and C, and 0.1 cm in D.

storage temperatures of vernalized bulbs or by spraying water or BAP solution to the inflorescences or by previous elimination of the floral buds (Matsubara and Hihara 1978; Brewster 1994).

The *in vitro* shoot proliferation by direct organogenesis from inflorescences has also been informed (Matsubara and Hihara 1978). In fact, it was possible to induce the production of multiple shoots from inflorescences in an early stage of development, i.e. with the first flowers in primordial stage, of the 'Valcatorce INTA' cultivar. For this approach, vernalized bulbs were cultivated under conditions of floral development (Brewster 1994). Then, umbel primordia of 1.2-2 mm, 2-4 mm and 4-6 mm wide (Fig. 1A) were cultivated in BDS medium with different concentrations of NAA and BAP. An average of 4.6 to 19.2 shoots per explant was obtained, although the wider range was between 0 and 58 shoots per explant. The higher number of microshoots was obtained in the case of umbels of higher size with the culture medium supplemented with 1 mg.L⁻¹ of both NAA and BAP (Fig. 1). Histological analysis showed that the microshoots differentiated from primordia were originated in the

umbel receptacle that still remained meristematic (Fig. 1D). Simultaneously, it was observed a low number of floral primordia in an advanced state of differentiation, which finally produced rudimentary flowers.

Callus induction and plant regeneration from local onion cultivars

Plantlet regeneration via indirect organogenesis was already informed for onion (Van der Valk *et al.* 1992; Juntawong *et al.* 1993; Zheng *et al.* 1998). Calli were induced *in vitro* from different explants, e.g., root segments, basal disc, leaves, IU and ovules (Dunstan and Short 1977; Van der Valk *et al.* 1992; Juntawong *et al.* 1993; Tanikawa *et al.* 1998, 2004).

Picloram, 2,4-D (2,4-dichlorophenoxyacetic acid) and dicamba were evaluated at different concentrations by their ability to induce calli from onion explants (Phillips and Luteyn 1983; Van der Valk *et al.* 1992; Juntawong *et al.* 1993), and the different responses depended on the auxin type, its concentration and genotype. The widely used auxin for calli induction is 2,4-D, in the range of 0.5 and 2 mg.L⁻¹ (Van der Valk *et al.* 1992; Zheng *et al.* 1998). Phillips and Luteyn (1983) recommended 0.75 mg.L⁻¹ picloram for calli induction while Tanikawa *et al.* (1998) found high regeneration levels from calli induced with 8.5 mg.L⁻¹ 4-fluorophenoacetic acid (4-FPA).

Marinangeli *et al.* (2005) reported on the regeneration capacity of calli induced from different explants of 'Valcatorce INTA' onion cultivar under identical cultural conditions. Qualitative and quantitative responses were studied in calli induction from different explants cultivated in different basal nutrient media, auxin types, auxin/cytokinin ratios and cultural conditions. The subsequent influence of these factors was evaluated, together with the effect of different cytokinins on plant regeneration. In order to compare the genotype effect, other *A. cepa* cultivars together with *A. fistulosum* were also evaluated using the same conditions for calli induction and shoot regeneration.

For 'Valcatorce INTA', the basal plate of the bulb with the apical meristem (BPM) and without (BP) the apical meristem, IU, floral scapes (FS), fecundated ovules (FO) and IZE from vernalized bulbs cultivated under conditions of floral development (Brewster 1994), as well as MZE obtained from seeds, were cultivated in darkness. For calli induction, BDS (Dunstan and Short 1977) or MS (Murashige and Skoog 1962) basal media were used with combinations of 2,4-D or picloram with BAP, and culture was done either in darkness or light. For the regenerating phase, calli were cultivated under light in BDS nutrient medium with BAP, Kinetin or 2iP (6-(γ - γ -dimethylallyl)-amino)-purine).

It was possible to induce calli from all the explants and with all the PGR concentration ratios, although the response was too variable and low in some cases (Fig. 2). The calli originated from IZE, MZE and FO were not compact, and consisted of small globular aggregates exhibiting a high growth rate, although the proportion of calli induction of FO was low (<33%). The M explants produced sprouts with leaves and the BP explants had a high proportion of contamination. The calli originated from these explants were mostly of the compact type. The calli originated from IU and FS were compact.

The calli induced with 2,4-D were mostly friable and composed by small globular aggregates (Fig. 3A, 3C). Picloram induced more compact and vitreous calli (Fig. 3B, 3D) that frequently differentiated hyperhydric roots in the successive subcultures (Fig. 3F). This phenomenon could not be reverted into the formation of shoots during the regeneration phase.

Shoots regeneration was low, 6.1% with kinetin, 6.3% with 2iP and 7.3% with BAP, in comparison to the results reported for other onion cultivars using the same explant types (Van der Valk *et al.* 1992; Zheng *et al.* 1998). The IZE explants produced the highest proportion of regenerant calli (87%) with two to eight shoots per callus; while BPM and

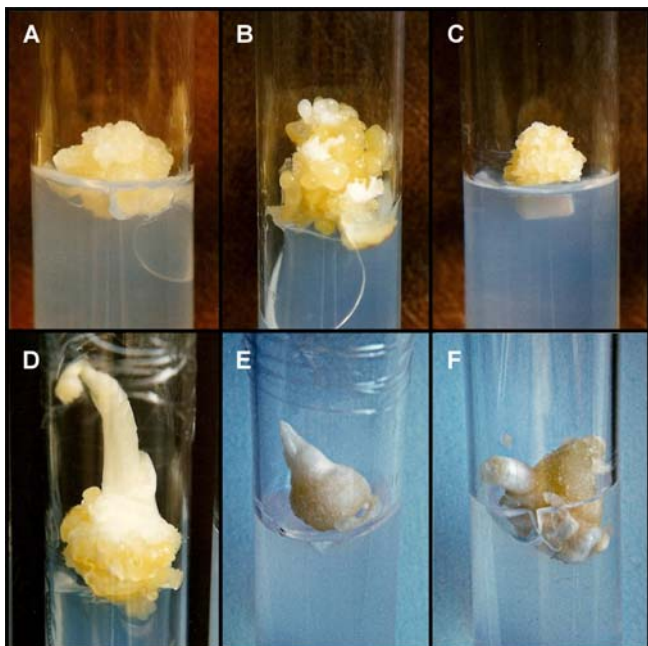


Fig. 2 Calli induced in BDS medium from different explant types of 'Valcatorce INTA' onion. BDS medium included 2 mg.L⁻¹ 2,4-D and 0.1 mg.L⁻¹ BAP. (A) Mature zygotic embryos, (B) fecundated ovules, (C) basal plate, (D) basal plate with apical meristem, (E) immature inflorescences and (F) floral scapes.

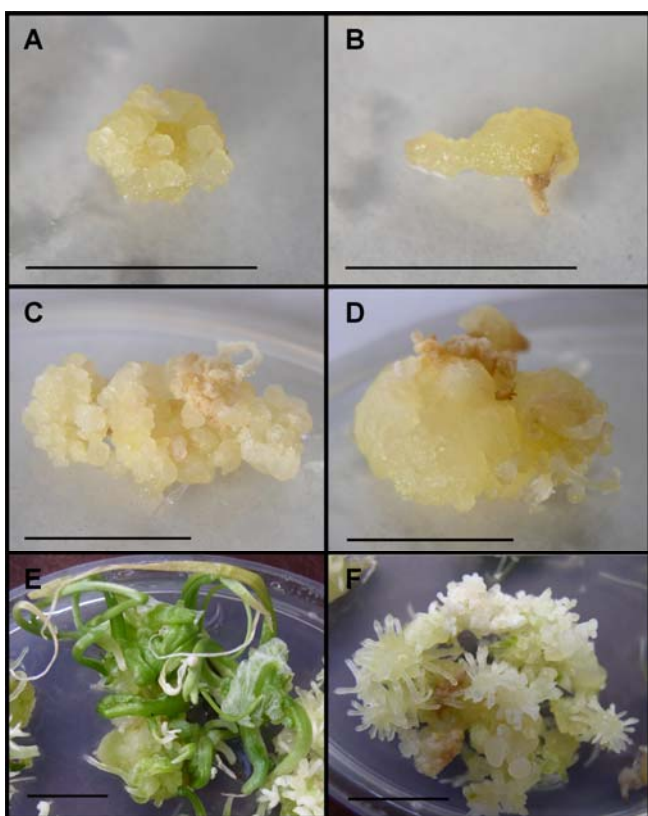


Fig. 3 Calli induced from mature zygotic embryos of 'Valcatorce INTA' onion and regenerated organs. (A) friable callus induced after 30 days in BDS medium with 2,4-D, (B) compact callus induced after 30 days in BDS medium with picloram, (C) friable callus induced after 60 days in BDS medium with 2,4-D, (D) compact callus induced after 60 days in BDS medium with picloram, (E) shoot regeneration from calli like C, and (F) root-type organs regenerated from calli like D. Scale: bars represent one cm.

BP did not formed shoots at all or it produced them at low rates. The auxin 2,4-D induced a higher proportion of regenerating calli; however, Phillips and Luteyn (1983)

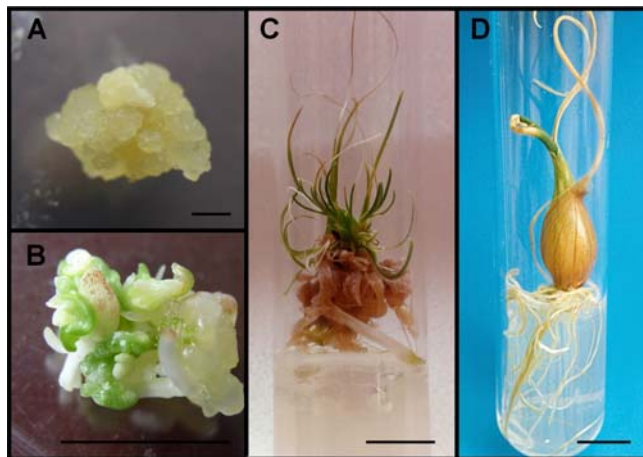


Fig. 4 Calli induction and plant regeneration of 'Valcatorce INTA' onion from mature zygotic embryos. (A) callus piece after 90 days of cultivation in induction medium (BDS + 2 mg.L⁻¹ 2,4-D), (B) shoot regeneration after 30 days of cultivation in regeneration medium (BDS + 0.1 mg.L⁻¹ BAP), (C) shoot regeneration after 60 days in regeneration medium, (D) microbulb produced in bulbing medium (BDS + 90 g.L⁻¹ sucrose). Scale: bar in A represents 1 mm, bar in B, C and D represents 1 cm.

informed a higher regeneration ratio from calli induced with picloram, although in that experience NAA also formed part of the nutrient medium.

During the regeneration phase, a high proportion of calli produced abundant quantity of roots as the only organogenic response; phenomenon that was mainly observed in calli induced with picloram, irrespective of the type of the initial explant and of the regeneration conditions (Fig. 3F).

When evaluating the induction of calli from MZE in both nutrient media, i.e. basal BDS vs. MS, it was found that the BDS medium produced calli of higher growth and higher regeneration ratio when 2,4-D was the auxin included. There were not differences in the proportion of regenerating calli when the induction was done either in light or in darkness.

MZE from four cultivars of *Allium cepa*, i.e. 'Valcatorce INTA', 'Norstar', 'T-412' (Takii) and 'Granex 33' (Asgrow Seeds), and from two varieties of *A. fistulosum*: 'Nogiwa Negi' and a wild type, were evaluated for callus induction and regeneration. Calli were induced in BDS medium containing 1 mg.L⁻¹ 2,4-D and 0.1 mg.L⁻¹ BAP in darkness, and for the regeneration phase they were cultivated in BDS with 0.1 mg.L⁻¹ BAP. All materials performed well during callus induction, reaching values near to 100%. Regeneration was dependent on the genotype and it was more variable among the *A. cepa* cultivars than among the varieties of *A. fistulosum*. The highest regeneration was obtained with *A. fistulosum* and T412 (ca. 42% of calli producing shoots) while the less regenerating genotypes were 'Valcatorce INTA' and 'Granex 33' (6.5%). These results agree with those reported by other researchers who also found a great dependence with the genotype in shoot regeneration efficiency from calli of *Allium cepa* (Phillips and Luteyn 1983; Zheng *et al.* 1998).

It was possible to induce calli from all the explants of 'Valcatorce INTA' and with all the evaluated PGRs. This also occurred with the other genotypes of *A. cepa* and *A. fistulosum*. However, a great variability in the response was found. This variability was higher among explants types than among different cultural conditions, and it appeared mainly in the regeneration phase. These results are in good agreement with the other ones reported for these species (Phillips and Luteyn 1983; Van der Valk *et al.* 1992; Tanikawa *et al.* 1998; Zheng *et al.* 1998). It is important to emphasize the poor performance exhibited by 'Valcatorce INTA' in the regeneration phase, with values lower than 10% of regenerating calli, except when these came from

IZE.

Regarding the explant type, a great variability was obtained in the regeneration response, including a lack of regeneration from some of them (BP and BPM). It should then be remarked that for an efficient and high plant-regenerating protocol, the explant type is very important. In the last reported research in onion, MZE and IZE were used as explants (Zheng and Kik 2008). Thus, for 'Valcatorce INTA', IZE could be a good alternative to the MZE considering the high regeneration ratio of the former. However, the IZE are less convenient from the point of view of their availability, since they can only be obtained when the seeds are in formation, soon after the end of flowering which is a narrow window of opportunity during few days per year.

Picloram should not be the auxin of election for callus induction in 'Valcatorce INTA' because it produces an abundant formation of roots during calli proliferation and regeneration phases, without shoots formation. On the other hand, 2,4-D was the auxin that induced calli with the highest rate of plantlet regeneration (Fig. 4).

It was possible to recover microbulbs from microshoots by cultivating them in BDS medium with higher rates of sucrose (90 g.L⁻¹) and without PGRs (Fig. 4D). Microbulbs are naturally resistant organs and they grew and flowered under greenhouse conditions.

CONCLUDING REMARKS

Although the multiplication ratio by direct organogenesis of the local onion cultivar 'Valcatorce INTA' was high from both MZE and IU, the use of these explants as target for transformation would have the risk of producing chimerical transgenic plants due to the regeneration from a group of cells. Since the transfection of DNA is an event limited to isolated cells, it is required that the regenerated plant be derived from the singles cells that has integrated the foreign DNA. Thus, with the direct regeneration pathway, an extra difficulty would exist during the *in vitro* selection phase, i.e. obtaining pure transgenic plants from a chimerical plant. Eventually, the risk exists not only of obtaining a chimerical plant for the transgene, but of even losing the transformation event during selection or by cellular competition. Also, in the case of IU, an additional difficulty is their seasonal availability.

Even when it was possible to obtain plantlets from calli of the 'Valcatorce INTA' onion cultivar, the efficiency was low. It was concluded that a regenerating system must consider the following: a) explants taken during the reproductive phase, i.e. IU, FO and IZE, b) use of BDS basal medium, and c) use 2,4-D as auxin for calli induction.

The restricted availability of explants in the reproductive stage during the year would limit their use in transformation and, for this reason the use of MZE obtained from seeds would be an option, although the regeneration rate was relatively low this being a limiting factor for the process of genetic transformation.

The high rate of shoot proliferation from IU cultivated *in vitro* suggests that the success of *in planta* transformation in onion is possible. As it was mentioned by Brewster (1994), differentiation of plantlets directly from inflorescences is possible; then, the *in planta* transformation of the juvenile umbel at the moment in which it appears among the leaves would be an appropriate way to introduce the DNA of interest, being it likely to be integrated to one of the produced bulblets or plantlets.

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REFERENCES

- Aswath C, Mo S, Kim D, Park S (2006) *Agrobacterium* and biolistic transformation of onion using non-antibiotic selection marker phosphomannose isomerase. *Plant Cell Reports* **25**, 92-99
- Barrueto Cid L, Illg RD, Piedrabuena AE (1994) Regeneration of garlic plants (*Allium sativum* L., cv. "Chonan") via cell culture in liquid medium. *In Vitro Cellular and Developmental Biology - Plant* **30**, 150-155
- Bent A (2000) Arabidopsis in plant transformation. Uses, mechanisms, and prospects for transformation of other species. *Plant Physiology* **124**, 1540-1547
- Brewster JJ (1994) *Onion and Other Vegetable Alliums*, CAB International, Cambridge, UK, 236 pp
- Birch RG (1997) Plant transformation: Problems and strategies for practical application. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 297-326
- Buitveld J (1994) Plant regeneration from protoplasts isolated from suspension cultures of leek (*Allium ampeloprasum* L.). *Plant Science (Limerik)* **100**, 203-210
- Campion B, Alloni C (1990) Induction of haploid plants in onion (*Allium cepa* L.) by *in vitro* culture of unpollinated ovules. *Plant Cell, Tissue and Organ Culture* **20**, 1-6
- Christou P (1997) Biotechnology applied to grain legumes. *Field Crop Research* **53**, 83-97
- Donovan A, Collin HA, Issac S, Mortimer AM (1994) Analysis of potential sources of variation in tissue culture derived celery plants. *Annals of Applied Biology* **124**, 383-398
- Dowker BD (1990) Onion breeding. In: Rabinowitch HD, Brewster JL (Eds) *Onions and Allied Crops* (Vol 1), CRC Press Inc., Florida, pp 215-232
- Dunstan DI, Short KC (1977) Improvement growth of tissue cultures of the onion, *Allium cepa*. *Physiologia Plantarum* **41**, 70-72
- Eady CC (1995) Towards the transformation of onions (*Allium cepa*). *New Zealand Journal of Crop and Horticultural Science* **23**, 239-250
- Eady CC, Butler R, Suo Y (1998) Somatic embryogenesis and plant regeneration from immature embryo cultures of onion (*Allium cepa*). *Plant Cell Reports* **18**, 111-116
- Eady CC, Weld RJ, Lister CE (2000) *Agrobacterium tumefaciens*-mediated transformation and transgenic-plant regeneration of onion (*Allium cepa* L.). *Plant Cell Reports* **19**, 376-381
- Eady CC (2002) Genetic transformation of onions. In: Rabinowitch HD, Currah L (Eds) *Allium Crop Science: Recent Advances*, CAB International, Wallingford, UK, pp 119-144
- Eady CC, Davis S, Farrant J, Reader J, Kenel F (2003a) *Agrobacterium tumefaciens*-mediated transformation and regeneration of herbicide resistant onion (*Allium cepa*) plants. *Annals of Applied Biology* **142**, 213-217
- Eady CC, Reader J, Davis S, Dale T (2003b) Inheritance and expression of introduced DNA in transgenic onion plants (*Allium cepa*). *Annals of Applied Biology* **142**, 219-224
- Eady CC, Davis S, Catanach A, Kenel F, Hunger S (2005) *Agrobacterium tumefaciens*-mediated transformation of leek (*Allium porrum*) and garlic (*Allium sativum*). *Plant Cell Reports* **24**, 209-215
- Fellner M, Havranek P (1994) Culture of protoplasts isolated from leaves and callus cultures of *Allium sativum* and *A. longicuspis*: A preliminary report. *Biologisches Zentralblatt* **113**, 317-328
- Galmarini CR (2000) Onion cultivars released by La Consulta Experimental Station, INTA, Argentina. *HortScience* **35**, 1360-1362
- Galmarini CR, Gaviola JC, Pereyra M, Burzichelli S, Ruiz AM (2003) Breve caracterización de la cadena cebolla en la Argentina. *IDIAXXI* **3**, 77-79
- Hanelt P (1990) Taxonomy, evolution and history. In: Rabinowitch HD, Brewster JL (Eds) *Onions and Allied Crops* (Vol 1), CRC Press Inc., Florida, pp 1-26
- Hansen G, Wright M (1999) Recent advances in the transformation of plants. *Trends in Plant Science* **4**, 226-231
- Hansen EE, Hubstenberger JF, Phillips GC (1995) Regeneration of shoots from cell suspension-derived protoplasts of *Allium cepa*. *Plant Cell Reports* **15**, 8-11
- Havel L, Novak FJ (1985) Meristem-tip culture of *Allium cepa* L. *Scientia Horticulturae* **27**, 209-214
- Hussey G, Falavigna A (1980) Origin and production of *in vitro* adventitious shoots in the onion, *Allium cepa* L. *Journal of Experimental Botany* **31**, 1675-1686
- Ikeda Y, Imoto M (1991) Production of virus-free plant of *Allium wakegi* Araki by means of shoot apex culture and its efficiency. *Bulletin of the Hiroshima Prefectural Agricultural Experiment Station* **54**, 41-46
- Juntawong N, Kaumanee P, Nanankorn M, Khewhok S (1993) Induction of onion (*Allium cepa* L. cv. Granex 33) callus formation and embryogenesis. *Kasetsart Journal Natural Science* **27**, 463-468
- Kahane R, Rancillac M, Teyssendier de la Serve B (1992a) Long-term multiplication of onion (*Allium cepa* L.) by cyclic shoot regeneration *in vitro*. *Plant Cell, Tissue and Organ Culture* **28**, 281-288
- Kahane R, Teyssendier de la Serve B, Rancillac M (1992b) Bulbing in long-day onion (*Allium cepa* L.) cultured *in vitro*: Comparison between sugar feeding and light induction. *Annals of Botany* **69**, 551-555

- Keller ERJ** (1993) Sucrose, cytokinin and ethylene influence formation of *in vitro* bulblets in onion and leek. *Genetic Resources and Crop Evolution* **40**, 113-120
- Kohmura H, Ikeda Y, Sakai A** (1994) Cryopreservation of apical meristems of Japanese shallot (*Allium wakegi* A.) by vitrification and subsequent high plant regeneration. *Cryo-Letters* **15**, 289-298
- Kumar KK, Maruthasalam S, Loganathan M, Sudhakar D, Balasubramanian P** (2005) An improved *Agrobacterium*-mediated transformation protocol for recalcitrant elite *indica* rice cultivars. *Plant Molecular Biology Reporter* **23**, 67-73
- Lydiate D, Dale P, Lagercrantz U, Parkin Y, Howell P** (1995) Selecting the optimum genetic background for transgenic varieties, with examples from *Brassica*. *Euphytica* **85**, 351-358
- Marinangeli PA, Zappacosta D, Galmarini CR, Curvetto NR** (2005) Callus induction and plant regeneration in onion (*Allium cepa* L.). *Acta Horticulturae* **688**, 301-308
- Marinangeli PA, Deluchi B, Ríos R, Franzone P, Galmarini CR, Rosselló F, Curvetto NR** (2006) Towards genetic transformation of local onion varieties. *Electronic Journal of Biotechnology* **9**, 331-334
- Martínez LE, Agüero CB, López ME, Galmarini CR** (2000) Improvement of *in vitro* gynogenesis induction in onion (*Allium cepa* L.) using polyamines. *Plant Science* **156**, 221-226
- Matsubara S, Hihara H** (1978) Onion bulblet regeneration on receptacles *in vivo* and *in vitro*. *Journal of the Japanese Society for Horticultural Science* **46**, 479-486
- Mohamed-Yasseen Y, Splittstoesser WE** (1992) Regeneration of onion (*Allium cepa*) bulbs *in vitro*. *PGRSA Quarterly* **20**, 76-82
- Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays in tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497
- Nomura Y, Makara K** (1993) Production of interspecific hybrids between rakkyo (*Allium chinense*) and some other *Allium* species by embryo rescue. *Japanese Journal of Breeding* **43**, 13-21
- Nomura Y, Kaeda M, Tsuchiya T, Makara K** (1994) Efficient production of interspecific hybrids between *Allium chinense* and edible *Allium* spp. through ovary culture and pollen storage. *Breeding Science* **44**, 151-155
- Novak FJ** (1990) *Allium* tissue culture. In: Rabinowitch HD, Brewster JL (Eds) *Onions and Allied Crops* (Vol 1), CRC Press Inc., Florida, pp 233-239
- Novak FJ, Havel L, Dolezel J** (1986) *Allium*. In: Evans DA, Sharp WR, Ammirato PV (Eds) *Handbook of Plant Cell Culture. Techniques and Applications* (Vol 4), Macmillan Publishing Co., NY, pp 419-456
- Pathak CS, Veere Gowda R** (1993) Improvement of onion. In: Chawda KL, Kallo G (Eds) *Advances in Horticulture* (Vol 5), Malhotra Publishing House, New Delhi, India, pp 181-99
- Phillips GC, Luteyn KJ** (1983) Effects of picloram and other auxins on onion tissue cultures. *Journal of the American Society for Horticultural Science* **108**, 948-943
- Pike LM, Yoo KS** (1990) A tissue culture technique for the clonal propagation of onion using immature flower buds. *Scientia Horticulturae* **45**, 31-36
- Ponce M, Martínez L, Galmarini CR** (2006) Influence of CCC, putrescine and gellam gum concentration on gynogenic embryo induction in *Allium cepa*. *Biologia Plantarum* **50**, 425-428
- Quintana-Sierra ME, Robledo-Paz A, Santacruz-Varela A, Gutiérrez-Espinosa MA, Carrillo-Castañeda G, Cabrera-Ponce JL** (2005) Regeneration *in vitro* of onion (*Allium cepa* L.) plants. *Agrociencia* **39**, 647-655
- Schum A, Mattiesch L, Timmann EM, Hofmann K** (1994) Regeneration of dihaploids via gynogenesis in *Allium porrum* L. *Gartenbauwissenschaft* **58**, 227-232
- Song P, Peffley EB** (1994) Plant regeneration from suspension cultures of *Allium fistulosum* and an *A. fistulosum* X *A. cepa* interspecific hybrid. *Plant Science* **98**, 63-68
- Tanikawa T, Takagi M, Ichii M** (1998) Varietal differences in plant regeneration *in vitro* of solid and suspension cultures in onion (*Allium cepa* L.). *Journal of the Japanese Society for Horticultural Science* **67**, 856-861
- Tanikawa T, Uragou H, Takagi M, Ichii M** (2004) Histological investigation of the plant regeneration process in onion (*Allium cepa* L.) calli. *Journal of the Japanese Society for Horticultural Science* **73**, 340-342
- Van der Valk P, Scholten OE, Verstappen F, Jansen RC, Dons JJM** (1992) High frequency somatic embryogenesis and plant regeneration from zygotic embryo-derived callus cultures of three *Allium* species. *Plant Cell, Tissue and Organ Culture* **30**, 181-191
- Xu QJ, Cui CR** (2007) Genetic transformation of OSISAP1 gene to onion (*Allium cepa* L.) mediated by a microprojectile bombardment. *Journal of Plant Physiology and Molecular Biology* **33**, 188-196
- Zheng SJ, Kik C** (2008) Recent developments and future prospects of gene transfer in *Allium* species. In: Rao GP, Zhao Y, Radchuk VV, Bhatnagar SK (Eds) *Advances in Plant Biotechnology*, Studium Press LLC, Texas, pp 457-473
- Zheng SJ, Henken B, Sofiari E, Jacobsen E, Krens F, Kik C** (1998) Factors influencing induction, propagation and regeneration of mature zygotic embryo-derived callus from *Allium cepa*. *Plant Cell, Tissue and Organ Culture* **53**, 99-105
- Zheng SJ, Henken B, Sofiari E, Keizer P, Jacobsen E, Kik C, Krens F** (1999) Effect of cytokinins and lines on plant regeneration from long-term callus an suspension cultures of *Allium cepa* L. *Euphytica* **108**, 83-90
- Zheng SJ, Khrustaleva L, Henken B, Sofiari E, Jacobsen E, Kik C, Krens F** (2001) *Agrobacterium tumefaciens*-mediated transformation of *Allium cepa* L.: The production of transgenic onions and shallots. *Molecular Breeding* **7**, 101-115
- Zheng SJ, Henken B, De Maagd RA, Purwito A, Krens F, Kik C** (2005) Two different *Bacillus thuringiensis* toxin genes confer resistance to beet armyworm (*Spodoptera exigua* Hübner) in transgenic *Bt*-shallots (*Allium cepa* L.). *Transgenic Research* **14**, 261-272