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-ami-no)-purine; NAA, α-naphtaleneacetic 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 resistance 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’, ‘Cobriza 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.* 2000). 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.

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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 (Lydiate 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.

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

<table>
<thead>
<tr>
<th>Horticultural Group</th>
<th>Cultivar/genotype</th>
<th>Target tissue</th>
<th>Transformation method</th>
<th>Expressed Gen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion</td>
<td>open-pollinated</td>
<td>immature zygotic embryos</td>
<td>Agrobacterium tumefaciens</td>
<td>nptII and gfp</td>
<td>Eady et al. 2000</td>
</tr>
<tr>
<td>‘Canterbury Longkeeper’</td>
<td></td>
<td>calli from mature zygotic embryos</td>
<td>A. tumefaciens</td>
<td>gusA and hpt</td>
<td>Zheng et al. 2001</td>
</tr>
<tr>
<td>‘Sturon’ and ‘Hyton’</td>
<td>open-pollinated Shallot</td>
<td>calli from mature zygotic embryos</td>
<td>A. tumefaciens</td>
<td>gusA and hpt</td>
<td>Zheng et al. 2001</td>
</tr>
<tr>
<td>‘Tropix’ and ‘Kuning’</td>
<td>Shallot</td>
<td>calli from mature zygotic embryos</td>
<td>Microprojectile acceleration</td>
<td>gusA, hpt and Bt</td>
<td>Zheng et al. 2005</td>
</tr>
<tr>
<td>‘Canterbury Longkeeper’</td>
<td>open-pollinated Onion</td>
<td>immature zygotic embryos</td>
<td>A. tumefaciens</td>
<td>GFP and nptII</td>
<td>Eady et al. 2005</td>
</tr>
<tr>
<td>inbred line KU-31</td>
<td>Onion</td>
<td>calli from seed radicles</td>
<td>Microprojectile acceleration and A. tumefaciens</td>
<td>Positive selectable marker pmi</td>
<td>Aswath et al. 2006</td>
</tr>
<tr>
<td>HG400B</td>
<td>Onion</td>
<td>calli from stem discs</td>
<td>Microprojectile acceleration</td>
<td>OSISAP1 (Oryza sativa stress-associated protein gene)</td>
<td>Xu and Cui 2007</td>
</tr>
</tbody>
</table>

* According to Hanelt (1990)

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 Argentinian 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 auxiliary 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 callus 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 Splitsstoesser 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 Splitsstoesser 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 (Bohance 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 Splitsstoesser 1992; Keller 1993), basal parts (Young et al. 1978), bulb sections (Dunstan and Short 1977; Hussey and Falavigna 1980), immature floral buds (Pike and Yoo 1990; Martinez 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 if the red/far-red ratio of light irradiation and the photoperiod 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 supplemented with PGRs to produce calli with differentiated tissues. If these calli are non differentiated and therefore, the establishment of cell suspensions culture protocol for regeneration of onion plants is a prerequisite 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. After 10 months of 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

Table 1
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 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-fluoroophenoxyacetic acid (4-FPA).

Marianegili 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-(/g534-/g534-dimethylallyl- amino)-purine). It was possible to induce shoots 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 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 all the PGR concentration ratios, although the response was too variable and low in some cases (Fig. 2).

Phosphorus and NAA (1 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.

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**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).
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BP did not form 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) 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’, ‘Nortstar’, ‘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
IRE.

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 Kk 2008). Thus, for ‘Valacorta 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 ‘Valacorta INTA’ because it produces an abundant formation of roots during callus 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 BDT medium with higher rates of sucrose (90 g•L−1) and without PGIs (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 ‘Valacorta INTA’ was high from both MZE and IU, the use of these explants as target for transformation would have the risk of producing chimeral 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 chimeral plant. Eventually, the risk exists not only of obtaining a chimerical plant for the transgene, but of even loosing 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 ‘Valacorta 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.

ACKNOWLEDGEMENTS

The own research referred by the authors was financed by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional del Sur and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT-FONCyT) of Argentina. The authors gratefully acknowledge Silvia Delmastro for the critical reading of the manuscript.

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