Improvement of regeneration in pepper: a recalcitrant species

Najet Gammoudi[1], Tania San Pedro[2], Ali Ferchichi[1], Carmina Gisbert[3*]

[1]Arid and Oases Cropping Laboratory, Arid Lands Institute (IRA), 4119 Medenine, Tunisia

[2] Instituto Valenciano de Investigaciones Agrarias (IVIA), Centro de Protección Vegetal y Biotecnología, Carretera de Moncada a Náquera km 4.5, 46113 Moncada, Spain

[3] Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV). Universitat Politècnica de València, Camino de Vera, 14 46022 Valencia, Spain

*Corresponding author: cgisbert@btc.upv.es

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Abstract

Organogenesis is influenced by factors like genotype, type of explant, culture medium components, and incubation conditions. The influence of ethylene, which can be produced in the culture process, can also be a liming factor in recalcitrant species like pepper. In this work, bud induction was achieved from cotyledons and hypocotyls - from eight pepper cultivars - on Murashige and Skoog (MS) medium supplemented with 22.2 µM 6-benzyladenine (6BA) and 5.7 µM indole-3-acetic acid (IAA), in media with or without silver nitrate (SN) (58.9 µM), a suppressor of ethylene action. In the SN-supplemented medium, the frequencies of explants with buds and with callus formation were lower in both kinds of explant, but higher numbers of developed shoots were isolated from explants cultured on SN. Bud elongation was better in medium with gibberellic acid (GA_3) (2.9 µM) than in medium free of growth regulators or supplemented with 1-aminocyclopropane-1-carboxylic acid (ACC) at 34.5 µM. However, isolation of shoots was difficult and few plants were recovered. The effect of adding SN following bud induction (at 7 d) and that of dark incubation (the first 7 d of culture) was also assessed in order to improve the previous results. When SN was added after bud induction, similar percentages of bud induction were found for cotyledons (average frequency 89.4% without SN and 94.4% with SN) whereas they doubled in hypocotyls (50 % without SN and 87.7% with SN). In addition, in both kinds of explant, the number of developed plants able to be transferred to soil (developed and rooted) was greatly increased by SN. Dark incubation does not seem to improve organogenesis in pepper, and hypocotyl explants clearly represent a better explant choice - with respect to cotyledonary explants - for the pepper cultivars assayed.

Keywords: *Capsicum*, ethylene, organogenesis, $AgNO_3$ (SN), ACC

Introduction

Plant regeneration from *in vitro*-cultured explants which is required for various *in vitro* culture techniques (genetic transformation, protoplast fusion or micropropagation) has been described for a large number of crops (Brown and Thorpe 1995; De Filippis 2014). . However, the genus *Capsicum*, especially bell pepper, is considered recalcitrant for *in vitro* regeneration and genetic transformation (Liu *et al.* 1990; Steinitz *et al.* 1999; Kothari *et al.* 2010; Maligeppagol *et al.* 2016). It is reported that successful regeneration in pepper depends on the genotype, nutrient medium composition, concentration and combination of growth regulators, light and temperature regimes in the growth chamber, and type of explant (Dabauza and Peña 2001; Ochoa-Alejo and Ramirez-Malagon 2001). In addition, the recalcitrance in *Capsicum* has been correlated with the role of ethylene during growth and development (Santana-Buzzy *et al.* 2006). This gas may be produced endogenously and/or as the consequence of explant isolation when cutting (Kumar *et al.* 1998; Moshkov *et al.* 2008) and can influence regeneration positively or negatively in different species (González *et al.* 1997; Trujillo-Moya and Gisbert 2012; Tamimi 2015). The use of silver nitrate (SN), which inhibits ethylene action, for induction of pepper regeneration has been found to have a positive effect on elongation. However, most of the protocols established gave low efficiencies and scarce reproducibility. This has contributed to limited success in pepper transgenic transformation that is also common in other species of the *Solanaceae* like tomato or eggplant. Application of the recently reported technology of genome editing will increase the interest in breeding plants using this methodology (Bortesi and Fischer 2015). In fact, attempts in solanaceous crops like tomato (Brooks *et al.* 2014) and potato (Sawai *et al.* 2014) have been reported. The development of protocols with efficient regeneration is still necessary for pepper cultivars since it is a key step in this new technology.

The aim of this study was to investigate the influence of ethylene on bud induction and shoot development in cotyledonary and hypocotyl explants of eight pepper cultivars from Tunisia and Spain. For

this purpose we evaluated, in a first assay, the effect of adding SN to the shoot induction medium (SIM) and of three sets of conditions on plant elongation. In a second assay, we studied the effect of adding SN at the beginning of culture or after bud induction, as well as the effect of an initial dark incubation of the explants. The development of induced shoots was followed since the plants were able to be transferred to pots.

Materials and methods

Plant material and culture conditions. Eight pepper cultivars were used in this study; six from the South of Tunisia (cv.25, cv.27, cv.28, cv.31, cv.32, and cv.34) and two from Valencia (VCA-116 and AVA-8). These materials were obtained, respectively, from the collection held by the Arid and Oasis Cropping Laboratory (Arid Lands Institute, Tunisia) and from the collection of the Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV, Spain). The Tunisian cultivar 28, from the provenance of Maghraoua, has been previously studied and showed high tolerance of salinity; thus, it was subsequently selected for use in genetic improvement programs (Gammoudi *et al.* 2016). The Spanish cultivars showed resistance to *Meloidogyne incognita* in naturally infected soils (data not shown).

Seeds of the pepper cultivars were surface-sterilized by immersion for 10 min in a solution of 30% commercial bleach (containing 40 g L^{-1} of active chloride), followed by three rinses in sterile distilled water, and were cultured in plastic Petri dishes (90 x 25 mm) containing Murashige and Skoog (1962) basal medium (BM) with MS salts and vitamins (Murashige and Skoog 1962) (Duchefa, Haarlem, The Netherlands), 1.5% sucrose, and 0.7% plant agar (Duchefa). The pH of the medium was adjusted to 5.8 before autoclaving for 20 min at 121°C. After germination, cotyledons and hypocotyls were used as explants.

Culture of cotyledon and hypocotyl explants on SIM or SIM supplemented with SN. Explants were cultured on shoot induction medium (SIM) (MS including vitamins, 3% sucrose, 0.7% plant agar, 22.2 µM 6-benzyladenine (6BA), and 5.7 µM indole-3- acetic acid (IAA)) and on SIM supplemented with SN (58.9

µM). Sixteen plates with cotyledon explants and eight plates with hypocotyls were cultured for each treatment, for 15 d. After this period, the explants of each treatment were divided into three groups and transferred to BM, BM supplemented with 34.5 µM 1- aminocyclopropane- 1 -carboxylic acid (ACC), or BM containing 2.9 µM gibberellic acid (GA_3). In these media, the explants were cultured for another 20 d. After this period, explants with leaves were transferred to tubes with BM and GA_3 (2.9 µM) for two mo.

All these compounds - 6BA, SN, ACC, and GA_3 - were filter-sterilized (0.20 µm; Syringe filters, LLG Labware, Germany) and then added to sterilized SIM medium. The media were plated in Petri dishes (90 x 15 mm) with 30 ml of culture medium (plates used for the first 15 d of culture), or in 90 x 25 mm plates with 40 ml of medium per plate (used for the following 20 d of culture). The pH of all the media was adjusted to 5.8 before sterilization at 121ºC for 20 min, and the cultures were incubated in a growth chamber at 26ºC ± 2ºC under a 16 h photoperiod, with cool white light provided by Sylvania cool white F37T8/CW fluorescent lamps (90 µmol m^{-2} s^{-1}).

The frequency of explants with organogenic buds (B) and the frequency of shoot regeneration (R) were determined at 15 and 35 d of initial culture.

The effects of SN addition after bud induction and culture in the dark vs. standard conditions. Cultivars AVA-8, cv.27, cv.28, and cv.34 were used in this assay (these cultivars showed high B values and differences in R in the previous assay). Cotyledon (C) and hypocotyl (H) explants were obtained from disinfected and germinated seeds and were cultured on SIM or SIM supplemented with SN. Explants cultured on SIM were transferred to SIM containing SN after 7 d of culture (to compare the effects of adding SN at d 0 or after 7 d of culture). In addition, for each treatment, half of the explants were cultured under standard conditions (S) and the other half in the dark (D) for the first 7 d of culture. Five explants per plate and eight plates per cultivar and treatment (CS0, CS7, CD0, CD7, HS0, HS7, HD0, and HD7) were used. After 15 d of the initial culture, B was noted and the explants were subcultured to plates with BM and GA_3 (2.9 µM). Every 15 d for 2 mo, explants that showed developed leaves ('R') were transferred to tubes with GA_3 (1.4µM) and activated charcoal (AC) (0.4%). The number of healthy shoots developed was noted.

In the two experiments, the tubes were sealed tightly with cotton plugs that enabled aeration and gas exchange.

Statistical analysis. The data were subjected to a unifactorial and multifactorial analysis of variance (ANOVA) and the means for the different traits were subsequently separated by a Tukey test.

Results and Discussion

The most common growth regulators used to induce organogenesis in pepper, considered a recalcitrant species for *in vitro* culture, are combinations of BA with the auxin IAA (Gunay and Rao 1978; Christopher and Rajam 1996; Sanatombi and Sharma 2008). Other cytokinins, like zeatin or thidiazuron, have been used also, but low percentages of buds and plants -that did not elongate further - were obtained (Hyde and Phillips 1996). The addition to the medium of SN, to inhibit ethylene action, is also frequent (Santana-Buzzy *et al.* 2005, 2006; Ashrafuzzaman *et al.* 2009; Orlińska and Nowaczyk 2015). This gas accumulates in *in vitro* culture as a consequence of wounding and can affect growth, differentiation, and senescence in plants at concentrations as low as 0.01 µM (Reid 1995).

As a first step, the organogenesis response of cotyledonary and hypocotyl explants of eight pepper cultivars cultured on a SIM that contained BA and IAA, supplemented or not with SN, was determined. High percentages of bud induction (>93%), in both hypocotyls and cotyledons, were obtained in SIM (Fig. 1a and c; Table 1) whereas lower percentages were noted for explants cultured on SIM supplemented with SN (80.9% for cotyledonary explants, and 28.2% for hypocotyls). Callus formation was also lower for both kind of explants in medium supplemented with SN (Table 1). Buds were mainly formed directly from the explants independently of callus formation. The cultivars differed in bud production and callus formation in medium containing SN (Fig.1). In this medium, the B was highest in cv.34 (with 92.5% of organogenic cotyledons and 62.8-% of responding hypocotyls), and the lowest response was observed for cotyledonary explants of cv.27 (52.5%) and hypocotyls from cv.25 (5%). Differences among cultivars were also observed for callus formation in explants cultured on SN-containing medium (Fig.1); whereas in cotyledonary explants callus appeared in percentages higher than 48% in VCA-116, cv.28, cv.31, cv.32 and cv.34, in

hypocotyl explants the maximum C was 32.5% (in cv.27 and cv.31). Overall, after this culture period, the cotyledons had a higher percentage of responding explants than the hypocotyls (88.8% vs. 61%). This result is consistent with previous studies which reported cotyledons as more responsive than hypocotyls for regeneration in *Capsicum* spp. (Grozeva *et al.* 2012), although the contrary result was observed by Ashrafuzzaman *et al.* (2009). The lower percentages of explants with buds and callus formation in the medium containing SN may indicate that ethylene is involved on regeneration and cell division. Although differing effects of ethylene on *in vitro* plant regeneration have been reported, our result is in agreement with the work of Mantiri *et al.* (2008), who reported ethylene as necessary for somatic embryogenesis in *Medicago truncatula*, and that of Trujillo-Moya and Gisbert (2012), who indicated that it enhanced regeneration in the interspecific hybrid *Solanum lycopersicon* x *S. pennellii*. Also, the work of Yasmin *et al.* (2014) suggests that exogenous ethylene precursors need to be supplied to overcome low regeneration in rice harboring the *SUB1A* gene. The lower percentages of explants with callus as well as the lesser callus formation (visually assessed) in the SN-containing medium, with respect to SIM, especially in hypocotyl explants (Table 1; Fig. 2a, b vs. Fig. 2c, d), do not agree with the results in *Solanum lycopersicon* observed by Shah *et al.* (2014), who found higher callus induction in an SN-containing medium.

The elongation of buds from explants cultured on SIM and SIM supplemented with SN was studied by transferring them to BM or to BM supplemented with ACC or GA_3. Pepper elongation has been reported in a medium without regulators (Hyde and Phillips 1996) and in a GA_3-containing medium (Kumar *et al.* 2012). The addition of ACC was also evaluated because greater development of shoots was observed in *S. pennellii* plants cultured in a medium containing this precursor of ethylene (Trujillo-Moya and Gisbert 2012). After 20 d of culture, oxidation was observed in some cotyledonary explants from all tested cultivars, with higher proportions (around 25%) in those from cv.25 and cv.32 (Table 2; Fig. 2e). Oxidation was higher in media without SN (2.7 vs. 22.7). Only some hypocotyl explants (<8%) of cv.31 and cv.32 showed oxidation. The most oxidized cotyledonary explants were observed in the ACC-containing medium (32.5%). Despite this, on average, the percentage of regeneration (explants with shoots) in this medium was similar to that obtained in BM, and in both it was lower than that achieved in the medium supplemented

with GA_3 (33.5% in cotyledons and 62.6% in hypocotyls; Table 2). Therefore, in the pepper genotypes compared in this work, the supplementation of ethylene (at this dose) does not favor elongation, as occurred in *S. pennellii* (Trujillo-Moya and Gisbert 2012), and GA_3 gives better R.

In contrast to what occurred for bud induction, explants of cotyledons showed - on average - lower R than hypocotyl explants (20. 8% vs. 37.1%) and those from SIM supplemented with SN showed greater development than those from SIM: for cotyledons 3.1% vs. 38.4% and for hypocotyls 19.6% vs. 54.6%. Despite this positive effect on bud development of the inhibition of the ethylene effect, the formation of rosette-groups with well-developed leaves but without an apparent apex (mainly for hypocotyls, Fig. 2g) made the isolation of shoots difficult. This response occurred especially in genotype AVA-8. Mezghani *et al.* (2007) interpreted the low number of elongated plants found in their work as a consequence of the induction of teratological protuberances, structures that are frequently associated with fasciated and degenerative meristems. Whereas the leaves of shoots developed from hypocotyls had a good appearance (Fig. 2f), hyperhydricity of shoots and abnormal structures were frequently found in cotyledon explants. Among the cultivars, cv.31 showed the highest R rate of the cotyledonary explants (38.3%) with good response in hypocotyls (>50%) whereas AVA-8 and VCA-116 had, in average, the lowest R (≤20%).

The results obtained in the first experiment led us to compare explants cultured directly on SN-containing medium (0) with those transferred to SIM with SN after seven d (7) of bud induction - to test whether, with this procedure, organogenesis was not inhibited and elongation was improved. Also, we tested the putative influence of dark (D) incubation versus standard incubation (S). Dark incubation has been reported as positive for organogenesis induction in some species (Zhao *et al.* 2013; Carvalho *et al.* 2014). Four cultivars (AVA8, cv.27, cv.28, and cv.34) that showed high B values and differing R in the previous assay were compared in this assay. Among cultivars, higher B for cotyledonary explants was observed in cv.27 vs cv.34 or cv. AVA-8, and higher response was noted in hypocotyl explants from cv.28 respect those from cv.27. The cultivar AVA-8 showed the lowest callus formation for both kind of explants. A positive effect of transferring explants from SIM to SIM with SN after 7 d was observed in hypocotyl explants which increased B (from 69.9% to 90.6%). Higher C was also observed in these explants (Table

3). Dark incubation did not influence on B but lower C was produced in hypocotyl explants (Table 3). Therefore, when hypocotyls were cultured in the presence of the inhibitor of ethylene action, darkness may contribute to reduce cell division. All these results show the complexity of organogenesis, in which many interactions occur, as well as the differing responses of cells from cotyledons and hypocotyls.

Lastly, we evaluated the development of isolable shoots (that than can be cut from the explant) according to the treatment, after 60 d on BM supplemented with GA_3 in a medium solidified with agar and AC (Table 4; Fig. 2h). The number of developed shoots was highest in cv.28. The 132 isolated shoots had an average height of 1.5 cm at this time and 37.1% were well rooted (9.9 cm average root length). The number of developed shoots was lowest for AVA-8: 19 plants, 84.2% of them rooted and with an average height of 3.5 cm. Clearly, a higher number of isolable shoots was produced from hypocotyls with respect to cotyledons (185 vs. 35), and in a higher proportion from explants transferred to SIM+SN after 7 d of culture (128 vs. 92). In general, a positive effect of darkness on plant development or rooting was not observed, although in some treatments the number of developed plants obtained was double that from explants incubated under standard conditions (CS7 vs. CD7). In tobacco callus, Huxter *et al.* (1981) reported that more ethylene was produced during culture in dark conditions. Perhaps this could explain the greater regeneration from hypocotyls incubated under standard conditions in the present work. The number of developed plants was also higher in HD7 (vs. HD0) and CD7 (vs. CD0), which indicates a positive effect on development of the transfer of explants to SN-containing medium after induction. Although the rooting percentage differed among cultivars and treatments at the time of measurement, the results indicate that the medium used is adequate for the rooting of pepper plants of all the genotypes tested. This medium contains AC, which may adsorb inhibitory compounds and toxic metabolites and establishes a dark environment (Mohamed-Yasseen 2001; Nogueira *et al.* 2007). After rooting, the plantlets raised *in vitro* were successfully acclimatized (>96%) and grew well in the growth chamber (Fig. 2i).

Conclusions

Ethylene influenced pepper organogenesis induction in a different manner depending on the kind of explant. The addition of SN, a suppressor of ethylene action, at the start of the culture resulted in a lower percentage

of explants with visible buds, particularly for hypocotyl explants (the percentage of responding explants increased when SN was added after induction). Callus formation also decreased as a consequence of the inhibition of ethylene action, in both hypocotyl and cotyledonary explants, and was recovered when SN was applied in the second step. The genotype greatly influenced bud induction and shoot development, as did the protocol employed to induce organogenesis (explant, inducing medium, and culture conditions) and the composition of the culture medium used for elongation. Among the factors studied for their influence on elongation, GA_3 seems a good option to promote the elongation of induced buds whereas transfer to a medium without growth regulators or with ACC is not advisable. In general, dark incubation did not favor regeneration of pepper. A higher proportion of developed plants (able to be transferred to soil), that was the final aim of organogenic induction, was obtained from the explants when SN was added after inducement of organogenesis - probably a result of the increased number of buds in these explants and, putatively, their better development. Hypocotyl explants seem to represent a better explant choice than cotyledonary explants.

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