TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2014) 38: 465-476

doi:10.3906/bot-1305-16

Leaf morphology and shoot regeneration of in vitro cultured explants from species of the *Solanum peruvianum* s.l. complex

Carlos TRUJILLO-MOYA, Rosa PEIRÓ, Carmina GISBERT*
Center for the Conservation and Breeding of the Agricultural Biodiversity (COMAV), Polytechnic University of Valencia, Valencia, Spain

Received: 08.05.2013 • **Accepted:** 23.01.2014 • **Published Online:** 31.03.2014 • **Printed:** 30.04.2014

Abstract: We studied leaf morphology in in vitro plants of species segregated from the *Solanum peruvianum* L. s.l. complex: *S. arcanum* Peralta, *S. corneliomulleri* J.F.Macbr., *S. huaylasense* Peralta, and *S. peruvianum* L. s.str. In these species, the regeneration ability from root and leaf explants cultured in vitro in 2 organogenic media (SIM-1 and SIM-2) was also evaluated in a total of 16 accessions. These species can be differentiated by the number of leaflets, leaflet dentation of the in vitro cultured plants, and leaf area (for *S. arcanum*). Regarding regeneration, intraspecific and interspecific variability was observed. The accession LA-2185 of *S. arcanum* and the accessions ECU-106 and CH-20 of *S. peruvianum* s.str. can be considered low regenerating, whereas the rest of the accessions had a good and high regeneration capacity. Despite the 4 species being able to regenerate from root explants, leaf explants have been selected as more appropriate for in vitro regeneration. Better results were also obtained in the organogenic medium SIM-1 with respect to SIM-2. Interestingly, explants from leaves with a high amount and more dentate leaflets had high regeneration.

Key words: Arcanum, Eriopersicon, organogenesis, *Solanum*

1. Introduction

Wild tomatoes belong taxonomically to the genus *Solanum* L. section *Lycopersicon* (Mill.) Wettst. and include several useful species for breeders. The most recent classification of wild and cultivated tomatoes (Peralta et al., 2008) divided the section *Lycopersicon* into 4 groups: Lycopersicon group [*S. lycopersicum* L., *S. pimpinellifolium* L., *S. cheesmaniae* (L.Riley) Fosberg, and *S. galapagense* S.Darwin and Peralta], Neolycopersicon group (*S. pennellii* Correll), Eriopersicon group [*S. peruvianum* L., *S. corneliomulleri* J.F.Macbr., *S. huaylasense* Peralta, *S. habrochaites* S.Knapp and D.M.Spooner, and *S. chilense* (Dunal) Reiche], and Arcanum group [*S. arcanum* Peralta, *S. chmielewskii* (C.M.Rick, Kesicki, Fobes and M.Holle) D.M.Spooner, G.J.Anderson and R.K.Jansen, and *S. neorickii* D.M.Spooner, G.J.Anderson and R.K.Jansen]. Previously, *S. arcanum*, *S. corneliomulleri*, *S. huaylasense*, and *S. peruvianum* s.str. were included in the *S. peruvianum* s.l. complex (Peralta et al., 2005).

Based on RFLP, SSR, and AFLP data, the *S. peruvianum* s.l. complex is the most genetically variable in the genus and the most likely to contribute genes not currently found in cultivated tomato (Miller and Tanksley, 1990; Alvarez et al., 2001; Spooner et al., 2005; Zuriaga et al., 2009). For instance, resistance to *Begomovirus* and *Meloidogyne* spp. was found in *S. peruvianum* s.str. and *S. corneliomulleri* (Pereira-Carvalho et al., 2010), and resistance to *Alternaria solani* Sorauer (Chaerani et al., 2007), *A. tomatophila* Simmons (Foolad et al., 2007), and nematodes (Jablonska et al., 2007) in *S. arcanum* and *S. huaylasense*. However, the use of the *S. peruvianum* s.l. gene pool has been greatly limited due to incompatibility problems (Sacks et al., 1997; Bedinger et al., 2011). In order to solve this, in vitro culture techniques have been used for embryo rescue in crosses where embryos are formed but unable to develop. Actually, embryos that were developed to at least the heart-shaped stage have yielded seedlings via embryo culture (Smith, 1944; Cap et al., 1991) and callus culture has been used to obtain plants when embryos have not developed past the globular stage (Thomas and Pratt, 1981; Segeren et al., 1993). Protoplast fusion was also used to achieve hybrids between *S. peruvianum* s.l. and S. *lycopersicum* (Chen and Adachi, 1998; Kochevenko et al., 2000).

Regeneration from explants cultured in vitro is the starting point of most in vitro techniques. Thus, plant regeneration protocols have been described in tomato plants (Peres et al., 2001; Faria et al., 2002; Bhatia et al., 2004) and in the related species *S. peruvianum* s.l. (Koornneef et al., 1987), *S. hirsutum* Dunal. (currently *S. habrochaites*) (Stommel and Sinden, 1991), *S. pennellii* (Gisbert et al.,

* Correspondence: cgisbert@btc.upv.es

1999), *S. pimpinellifolium* (Pratta et al., 1997), *S. chilense* (Takashina et al., 1998), and *S. chesmanii* (Arrillaga et al., 2001) from different type of explants (cotyledonary, leaves, or roots). Plant regeneration via shoot organogenesis on decapitated seedlings was also studied in tomato plants and the related species *S. cheesmanii*, *S. chilense*, *S. chmielewskii*, *S. hirsutum*, *S. parviflorum* C.M.Rick, Kesicki, Fobes and M.Hole (currently *S. neorickii*), *S. peruvianum* s.l., and *S. pimpinellifolium* (Steinitz et al., 2006). In these studies, a great influence of genotype and intra- and interspecific variability of regeneration have been described. In general, high regeneration was observed in the wild species and recalcitrance in some tomato cultivars (Bhatia et al., 2004) and in *S. pimpinelifollium* (Marchionni et al., 2007). The presence of a gene (*Rg- 1*) that confers great regenerative capacity was described in *S. peruvianum* s.l. (Koornneef et al., 1993). Putative alleles of these genes were found in *S. chilense* (Satoh et al., 2000) and *S. pennellii* (Trujillo-Moya et al., 2011). Moreover, several QTLs for organogenic capacity were located in tomato (Trujillo-Moya et al., 2011), indicating that regeneration depends on several genes.

There is no information about in vitro regeneration in the 4 species segregated from the *S. peruvianum* s.l. complex: *S. arcanum*, *S. huaylasense*, *S. corneliomulleri*, and *S. peruvianum* s.str.. Thus, the aim of this study was to test the organogenic ability in accessions of interest that belong to these 4 species. For this purpose, we compared the organogenic capacity of 2 explant types in 2 organogenic-inducing culture media as well as the respective correlations with leaf morphology, which differs among these species.

2. Materials and methods

2.1. Plant material and growth conditions

We studied 16 accessions of 4 species (*S. peruvianum* s.str., *S. corneliomulleri*, *S. arcanum*, and *S. huaylasense*) that belong to the highly variable *S. peruvianum* s.l. complex (Table 1). All these accessions were supplied by Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV, Universitat Politècnica de València, Spain). Ten of these accessions were classified by Zuriaga et al. (2009) using morphological and molecular data (*S. huaylasense* (PE-18, PE-19, PE-20), *S. corneliomulleri* (T-040), *S. arcanum* (ECU-777, ECU-783, LA-2185), and *S. peruvianum* (CH-20, ECU-106, PER-412). The other 6 accessions (*S. huaylasense* (PE-27), *S. corneliomulleri* (PER-584, PER-586, PER-592) and *S. arcanum* (ECU-766, ECU-775) were classified just using the morphological data gathered in situ. The geographic areas of these accessions are shown in Figure 1.

Seeds were sterilized by immersion in a solution of 25% commercial bleach (40 g/L active chlorine) for 10 min, being then washed twice with sterile deionized water for 5 min each and then sown in petri dishes containing basal medium (BM:MS salts (Murashige and Skoog, 1962) including vitamins (DUCHEFA, the Netherlands), 1.5% sucrose and 7 g/L plant agar). The pH of the media was adjusted to 5.8 before sterilization at 121 °C for 20 min. Cultures were incubated in a growth chamber at 26 ± 2 °C under a 16-h photoperiod with cool white light provided by Sylvania cool white F37T8/CW fluorescent lamps (90 μmol m^{-2} s^{-1}).

Plants were maintained in vitro in tubes with BM. Every 3–4 weeks, plants were transferred to fresh medium. For each genotype, a population of 12 to 34 plants was established and used in our assays.

2.2. Evaluation of regeneration capacity

The evaluation was performed using root explants (0.5 cm in length; not including apical meristem) and leaf disks (0.6–0.8 cm^2) obtained from in vitro cultured plants at a similar growing stage. In each accession, leaf disks were obtained from leaves at the fourth and fifth position from the apex and cutting the extreme of each leaflet (Figure 2).

Explants were cultured onto 2 shoot induction media (SIM): SIM-1, containing MS salts including Nitch vitamins (DUCHEFA, the Netherlands), 3% sucrose, 7% plant agar, and 0.5 mg/L zeatin riboside; and SIM-2, which is similar to SIM-1 but supplemented with 0.2 mg/L indole-3-acetic acid. Leaf disks were placed with the abaxial side in contact with the SIM. Growth regulators were sterilized by filtration and added to the sterile SIM. For each accession, 5 explants per plate (90 × 25 mm with 40 mL of medium per plate) and 10 repetitions per accession were evaluated. After 35 days of culture on SIM, the following variables were analyzed:

- Bud percentage (B): number of explants with buds × 100/total number of cultured explants.
- Regeneration percentage (R): number of cultures that differentiated into completely developed shoots × 100/ total number of cultured explants.
- Productivity rate (PR): total number of completely developed shoots/total number of cultured explants that regenerated plants.
- Yield (Y): number of regenerated shoots/total number of cultured explants.

2.3 Evaluation of leaf shape

The number of leaflets (LN) in the fourth and fifth leaves was counted for each genotype. Leaflet area (LA) of the distal leaflet in these leaves was calculated. An index for leaflet margin dentation (LD) was also assigned: from 1 (low margin dentation) to 5 (high margin dentation). Duplicate measures were taken in 5 plants of each accession.

Table 1. Accessions used for the study of the regeneration capacity of the *Solanum peruvianum* L. s.l. complex (*S. huaylasense, S. corneliomulleri*, *S. arcanum*, and *S. peruvianum* s.str.), including accessions identifiers, country, region, and geographic coordinates for the collection sites.

Accession code	Country	Region	Latitude	Longitude
Solanum huaylasense				
PE-18 [a]	PER	Ancash	0849-S	07752-W
PE-19 [a]	PER	Ancash	0848-S	07752-W
PE-20 [a]	PER	Ancash	0848-S	07752-W
PE-27	PER	Ancash	0932-S	07750-W
Solanum corneliomulleri				
PER-584	PER	Arequipa	161125-S	0713920-W
PER-586	PER	Arequipa	161017-S	0713918-W
PER-592	PER	Arequipa	162116-S	0712959-W
T-040 [a]	PER	Ayacucho	144916-S	0744035-W
Solanum arcanum				
ECU-777 [a]	PER	Cajamarca	071817-S	0782839-W
ECU-766	PER	Cajamarca	071735-S	0783426-W
ECU-775	PER	Cajamarca	071710-S	0783015-W
ECU-783 [a]	PER	Cajamarca	071947-S	0781102-W
LA-2185 [a]	PER	Amazonas	052900-S	0783100-W
Solanum peruvianum				
CH-20 [a]	CHL	Tarapacá	182415-S	0695843-W
ECU-106 [a]	ECU	El Oro	0319-S	07930-W
PER-412 [a]	PER	Lima	115555-S	0763747-W

[a] Accessions used by Zuriaga et al. (2009). PER (Peru), CHL (Chile), and ECU (Ecuador).

2.4 Experimental design and statistical analysis

Least squares data analyses were performed on the regeneration capacity traits with the fixed effects of the species (*S. arcanum*, *S. corneliomulleri*, *S. huaylasense*, and *S. peruvianum* s.str.), the accession (16 levels and nested to the species effect), the medium (2 levels: SIM-1 and SIM-2), and the tissue (2 levels: leaf and root). The accession was nested to the species since the levels of one factor (accession) only make sense within the level of another factor (species). The leaf shape traits were analyzed with a mixed model that included a fixed effect of the species and accession (with the same levels described above) and a random effect of the measure. In the fixed effect analysis, we assume that the true effect size is the same in all studies, and the summary effect is our estimate of this common effect size. In the random effect analysis, we assume that the true effect size varies from one study to the next, and that the studies in our analysis represent a random sample of effect sizes that could have been observed. The summary effect is our estimate of the mean of these effects. For these reasons, the species and the accession effects were considered as fixed effects and the measure was considered as random effect. Correlations among regeneration and leaf shape traits and within them were analyzed excluding the main effects.

3. Results

3.1. Leaf morphology

Leaves with several leaflets, which differ in size and margin dentation, were observed in the accessions (Figure 2). There was a difference in the number of leaflets (LN) among the 4 species (Table 2); *S. arcanum* showed the lowest LN (with a mean of around 5 leaflets) and *S. corneliomulleri* showed the highest LN (with a mean higher than 8 leaflets). Leaflet area (LA) did not differ among *S. corneliomulleri*, *S. peruvianum* s.str., and *S. huaylasense*. These 3 species had lower LA than *S. arcanum*. Leaflet margin dentation

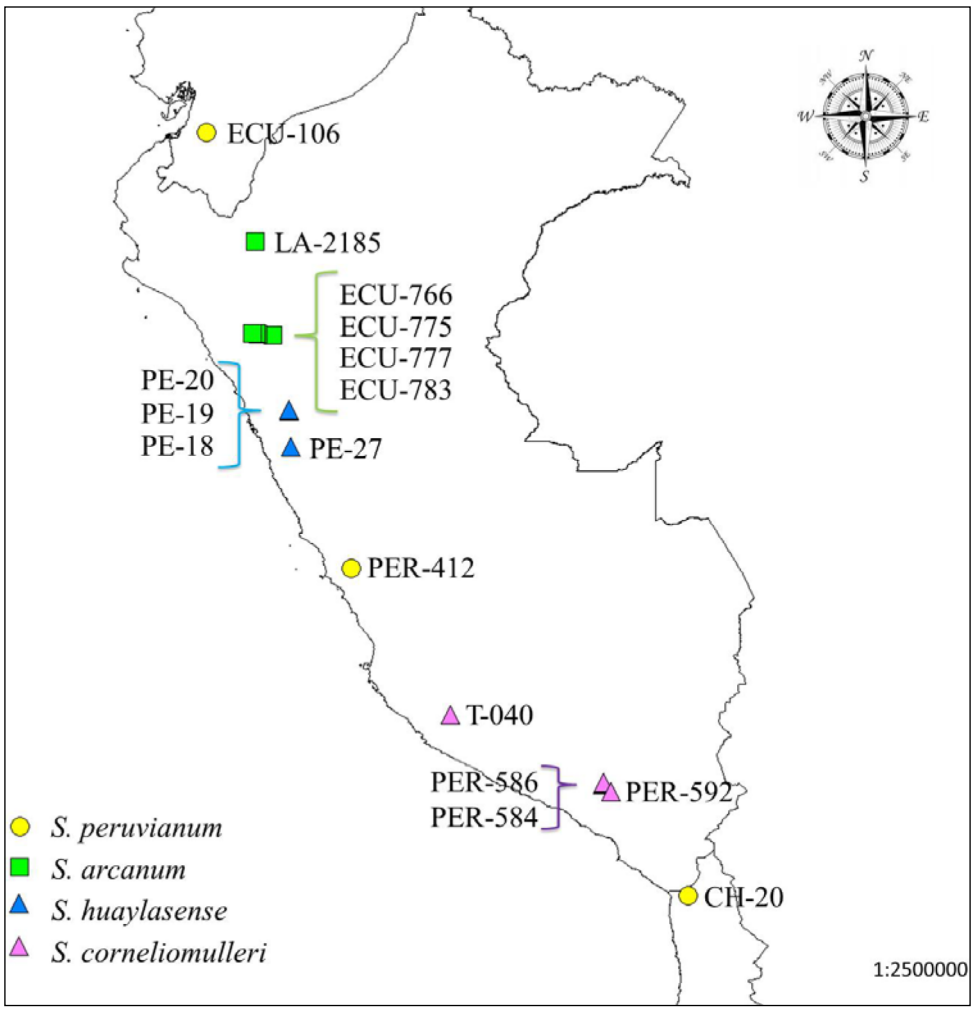

Figure 1. Geographic locations of the accessions studied here from southern Ecuador, Peru, and northern Chile.

(LD) showed the same pattern as number of leaflets; dentate margins increase successively (from 1.20 to 2.93) in *S. arcanum*, *S. peruvianum* s.str., *S. huaylasense*, and *S. corneliomulleri* (Figure 2; Table 2). Intraspecific variability is also showed for leaf morphology traits (Figure 3). The accession PER-592 had the highest number of leaflets (with a mean of 10.72) and margin dentation (with a mean of 4.50), whereas the accession LA-2185 had the highest leaflet area (with a mean of 1.82). Moreover, differences among accessions in *S. huaylasense* and S. *corneliomulleri* were found for the number of leaflets. Intraspecific differences were also found for leaflet area in S. *huaylasense* and *S. arcanum*. Finally, differences among accessions in all the species were found for leaflet margin dentation.

There was a positive correlation between NL and LD (0.54; Table 3). However, there were negative correlations among these traits and LA (–0.53 and –0.29, respectively).

3.2. Organogenesis

Intra- and interspecific variability in the organogenesis response was obtained from in vitro cultured explants (Tables 4 and 5; Figure 4).

The percentage of explants with buds (B) was higher in *S. corneliomulleri* (88.04%) than in the other species (from 63.82% to 78.11%; Table 4). *Solanum arcanum* and *S. peruvianum* s.str. showed the lowest B value. All the accessions were able to induce buds from leaf and root explants in both media (SIM-1 and SIM-2) except accession ECU-106, which only regenerated from leaf segments (Table 5). LA-2185 (*S. arcanum*) and ECU-106 (*S. peruvianum* s.str.) accessions showed the lowest B (Figure 4A). For this parameter, no or low differences among accessions were found in *S. corneliomulleri* and *S. huaylasense*, respectively.

The development of buds into shoots was measured as R (regeneration percentage). *Solanum huaylasense* and

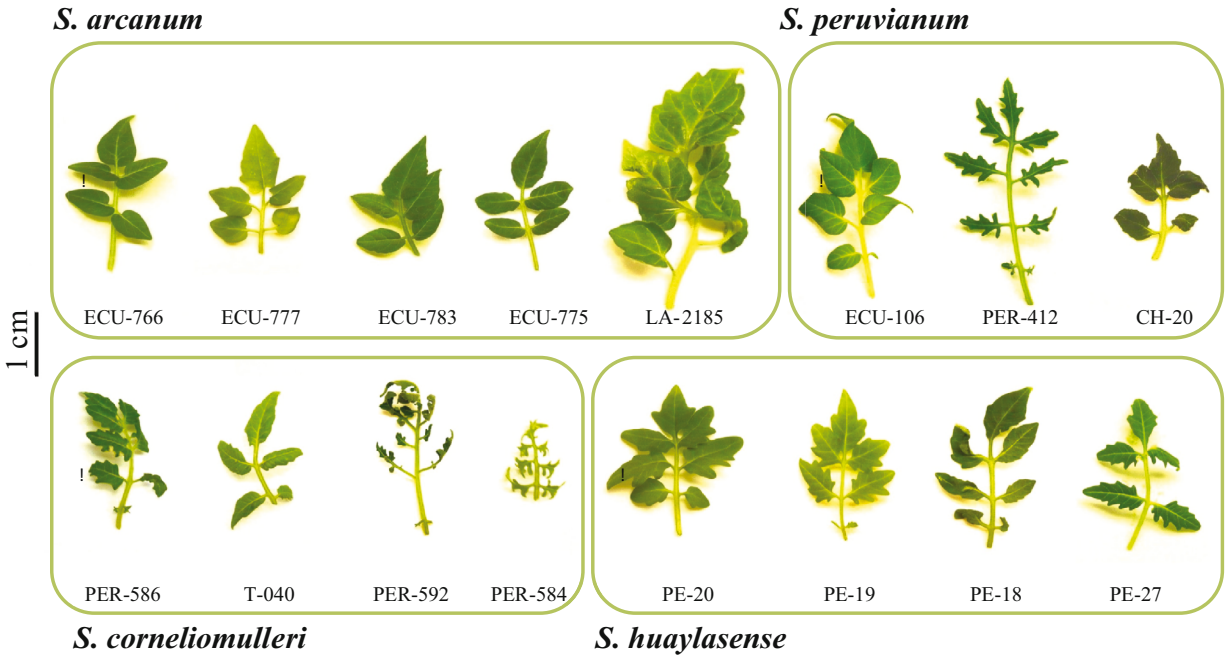

Figure 2. Leaf shape variability of *Solanum peruvianum* s.l. complex (*S. arcanum, S. peruvianum* (s.str.), *S. corneliomulleri*, and *S. huaylasense*), including accessions identifiers. Photos obtained from in vitro cultured plants at a similar growth stage.

S. corneliomulleri showed similar R values (51.69% and 46.70%, respectively) and both showed higher R values than *S. arcanum* (35.15%; Table 4). Variability among accessions for this trait was found in the 4 species. The lowest R value (around 10%) were obtained in accessions LA-2185 and ECU-106 of *S. arcanum* and *S. peruvianum* s.str., respectively, as occurred for the percentage of explants with buds (Figure 4B). The accession PER-412 of *S. peruvianum* s.str. showed the highest R (82.43%) and R values higher than 50% were obtained in PE-20 and PER-592 of *S. huaylasense* and *S. corneliomulleri*, respectively.

For productivity rate (PR) intra- and interspecific variability was also observed. For instance, *S. huaylasense* and *S. corneliomulleri* showed higher PR than *S. arcanum* and *S. peruvianum* s.str. (Table 4). Differences among accessions appeared in all species (Figure 4C). For example, in *S. huaylasense*, accession PE-20, which had the highest PR (with a mean of 8.10), differed from the other accessions (PE-18, PE-19, and PE-27), showing less than 7.

Plant regeneration per total explant considered as yield (Y) differed among species and accessions. *Solanum huaylasense* showed similar Y to *S. corneliomulleri* and both species showed higher Y values than *S. arcanum* (Table 4). Accession PE-20 of *S. huaylasense* showed the highest Y (with a mean of 7.95; Figure 4D). Among the other 3 accessions of *S. huaylasense*, Y values decreased successively in PE-18, PE-19, and PE- 27. In our study, *S. corneliomulleri* accessions collected in Arequipa region

Table 2. Least square means (±S.E.) for leaf morphology traits for *S. huaylasense, S. corneliomulleri, S. arcanum,* and *S. peruvianum* (s.str.). LN: Number of leaflets; LA: Leaflet area, LD: Leaflet margin dentation.

Factor	LN	LA	LD
S. huaylasense	7.45 ± 0.19 ^{b}	0.52 ± 0.07 ^{b}	2.35 ± 0.09 ^{b}
S. corneliomulleri	8.40 ± 0.19 ^{a}	0.53 ± 0.07 ^{b}	2.93 ± 0.09 ^{a}
S. arcanum	5.02 ± 0.17 ^{d}	1.15 ± 0.07 ^{a}	1.20 ± 0.08 ^{d}
S. peruvianum	6.10 ± 0.22 ^{c}	0.56 ± 0.09 ^{b}	1.93 ± 0.10 ^{c}

Means within a row with different superscript differ ($P < 0.05$) according to Duncan's test.

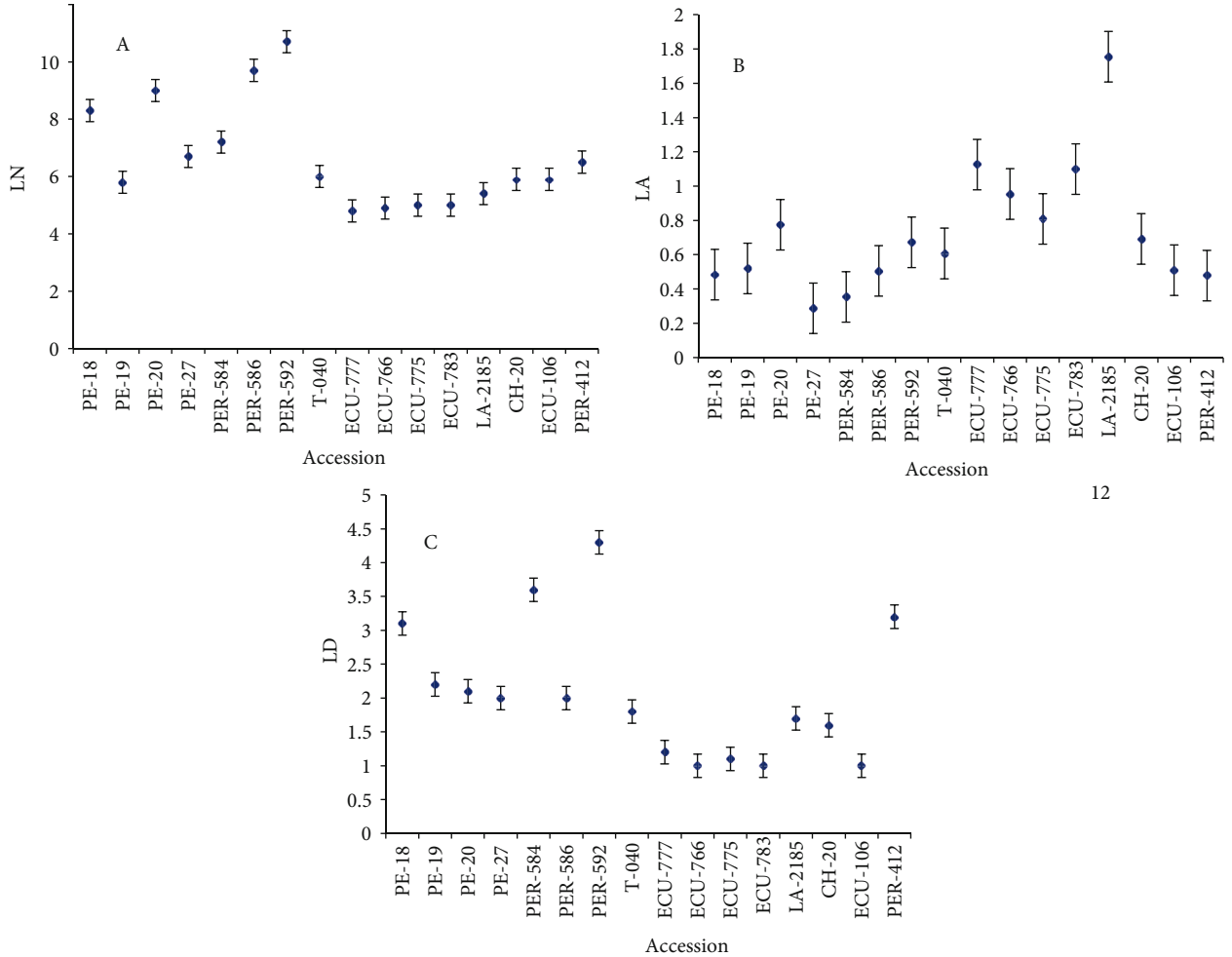

Figure 3. Means (±S.E.) for leaf morphology traits for accessions of *Solanum huaylasense*, *S. corneliomulleri*, *S. arcanum*, and *S. peruvianum* (s.str.). A- number of leaflets (LN), B- leaflet area (LA), and C- leaflet margin dentation (LD).

(PER-584, PER-586, and PER-592) showed similar Y values, whereas the accession collected in Ayacucho (TE-040) had a lower Y. Although *S. arcanum* showed the lowest Y value (1.67), accession PER-412 showed a Y value of 4, which is similar to the mean obtained in leaf explants. Taking into account Y values, the most recalcitrant accessions were CH-20 and ECU-106 of *S. peruvianum* s.str. and accession LA-2185 of *S. arcanum*.

Table 3. Correlation coefficients between residuals estimated for leaf morphology traits: number of leaflets (LN), leaflet margin dentation (LD), and leaflet area (LA).

	LD	LA
LN	0.54*	–0.22*
LD		0.29*

Different from zero *P < 0.05.

Differences between root and leaf explants as well as between SIM media (Table 4) were also observed; the organogenesis response was higher in leaf explants than in roots in all traits (89.74% vs. 57.71% for B, 64.51% vs. 23.37% for R, 4.94 vs. 2.51 for PR, and 4.20 vs. 0.74 for Y), whereas SIM-1 showed higher percentages of explants with buds (80.12%) and shoots (49.57%) than SIM-2 (67.33% and 38.31%, respectively).

Positive correlations among organogenic traits were found except for B and PR, where no correlation was obtained (Table 6). A high correlation (0.92) was observed between PR and Y.

Correlations among leaf morphology traits (LN, LA, and LD) and organogenic traits (B, R, PR, and Y) were also estimated (Table 7). There were positive correlations among LN and organogenic traits. Positive correlations were also found among LD and organogenic traits. However, negative correlations were found among LA and organogenic traits. All the correlations showed intermediate values.

Table 4. Least square means (±S.E.) for bud percentage (B), regeneration percentage (R), productivity rate (PR), and yield (Y) from in vitro cultured explants of *Solanum peruvianum* s.l. complex.

Factor	B	R	PR	Y
Species				
S. huaylasense	78.11 ± 3.19 [b]	46.70 ± 3.20 [a]	5.15 ± 0.48 [a]	3.86 ± 0.46 [a]
S. corneliomulleri	88.04 ± 3.24 [a]	51.69 ± 3.26 [a]	4.49 ± 0.46 [a]	3.03 ± 0.44 [ab]
S. arcanum	63.82 ± 2.95 [c]	35.15 ± 2.97 [b]	2.80 ± 0.57 [b]	1.67 ± 0.54 [c]
S. peruvianum	64.93 ± 3.87 [c]	42.23 ± 3.90 [ab]	2.46 ± 0.83 [b]	1.35 ± 0.78 [bc]
Explant				
Leaf	89.74 ± 2.37 [a]	64.51 ± 2.39 [a]	4.94 ± 0.35 [a]	4.20 ± 0.33 [a]
Root	57.71 ± 2.29 [b]	23.37 ± 2.31 [b]	2.51 ± 0.46 [b]	0.74 ± 0.44 [b]
Culture medium				
SIM-1	80.12 ± 2.36 [a]	49.57 ± 2.38 [a]	3.84 ± 0.37 [a]	2.62 ± 0.34 [a]
SIM-2	67.33 ± 2.31 [b]	38.31 ± 2.32 [b]	3.61 ± 0.44 [a]	2.33 ± 0.41 [a]

Means within a row with different superscript differ ($P < 0.05$) according to Duncan's test.

4. Discussion

4.1. Leaf morphology

Several traits related to leaves are commonly used in taxonomic classification (Peralta et al., 2005; Jiang et al., 2013). In our assay, we showed interspecific and intraspecific variability for leaf morphology and the species can be differentiated by number of leaflets or by leaflet margin dentation. However, leaflet area only separated *S. arcanum* from the other species. A putative explanation is that this trait may differ depending on culture conditions (in vitro vs. field). Pubescence, also used in tomato classification (Peralta et al., 2005), was not useful for differentiating species in plants cultured in vitro, although differences were evident in greenhouse conditions (data not shown). Some of the accessions used in our study were analyzed at molecular level using AFLP and 2 nuclear gene sequences and *S. arcanum* was separated from the other species included in the *S. peruvianum* s.l. complex (Zuriaga et al., 2009) as occurred with leaflet area in plants cultured in vitro (LA: 1.15 in *S. arcanum* vs. 0.55 approx. in the rest of the species).

Among *S. peruvianum* s.str. accessions, differences in leaflet margin dentation were found, whereas similar values were obtained for the other traits despite these accessions coming from different countries. Leaflet area and leaflet margin dentation were similar (around 1.0) in *S. arcanum* accessions collected at a similar zone (ECU-766, ECU-775, ECU-777, and ECU-783; Figure 1), whereas accession LA-2185, collected further north, showed large and high values (both around 1.8). In *S. huaylasense*, differences in all leaf morphology traits were found despite accessions being collected in the same region. Similar results were obtained for *S. corneliomulleri* accessions.

4.2. Organogenesis

Organogenesis was assayed in leaf and root explants cultured on SIM-1 and SIM-2. These explants were chosen because root segments are commonly used as explants for regeneration in *S. peruvianum* s.l. (Parker-Norton and Boll, 1954; Koornneef et al., 1993; Peres et al., 2001), whereas leaf explants are used for tomato plants (Kut and Evans, 1982; Ruf et al., 2001; Khoudi et al., 2009; Trujillo-Moya et al., 2011; Trujillo-Moya and Gisbert, 2012) and other related wild species (Gisbert et al., 1999; Arrillaga et al., 2001). Regarding shoot induction media, we used SIM-1, which is commonly used in our research group to induce organogenesis in both tomato and *S. pennellii* (Trujillo-Moya et al., 2011; Trujillo-Moya and Gisbert, 2012), and SIM-2, which is similar to SIM-1 but also contains the auxin indole-3-acetic acid. The combination of auxin and cytokinin was reported as favorable for regeneration in some tomato accessions (Bhatia et al., 2004; Devi et al., 2008).

Taking into account the results observed in the regeneration traits used for quantifying regeneration ability, we conclude that SIM-1 was better than SIM-2 for the percentages of explants with buds and shoots, whereas similar results were obtained for productivity rate and yield (Tables 4 and 5). Regeneration from roots was achieved in all accessions with the exception of accession ECU-106 of *S. peruvianum* s.str. (Table 5). Although regeneration in *S. peruvianum* s.l. was reported from root explants (Parker-Norton and Boll, 1954; Koornneef et al., 1993; Peres et al.,

Table 5. Accession means (±S.E.) for bud percentage (B), regeneration percentage (R), and productivity rate (PR) from in vitro cultured explants (L: leaf; R: root) on different shoot induction media (SIM-1, SIM-2).

Species	Accession	Explant	Media	B	R	PR
S. huaylasense	PE-18	L	SIM-1	100.00 ± 0.00	96.00 ± 4.00	10.42 ± 1.38
			SIM-2	88.00 ± 12.00	56.00 ± 14.70	2.79 ± 0.70
		R	SIM-1	48.00 ± 16.25	8.00 ± 8.00	7.25 ± 3.73
			SIM-2	52.00 ± 21.54	16.00 ± 11.66	3.50 ± 1.55
	PE-19	L	SIM-1	100.00 ± 0.00	96.00 ± 4.00	6.21 ± 0.76
			SIM-2	100.00 ± 0.00	52.00 ± 16.25	5.69 ± 1.53
		R	SIM-1	40.00 ± 18.97	8.00 ± 8.00	2.00 ±1.00
			SIM-2	40.00 ± 24.49	0.00 ± 0.00	0.00 ± 0.00
	PE-20	L	SIM-1	100.00 ± 0.00	100.00 ± 0.00	10.10 ± 1.37
			SIM-2	100.00 ± 0.00	100.00 ± 0.00	14.70 ± 2.03
		R	SIM-1	72.00 ± 17.44	52.00 ± 19.60	3.50 ± 1.04
			SIM-2	72.00 ± 17.44	28.00 ± 13.56	4.29 ± 0.64
	PE-27	L	SIM-1	100.00 ± 0.00	52.00 ± 10.20	3.77 ± 0.78
			SIM-2	96.00 ± 4.00	36.00 ± 11.66	1.56 ± 0.24
		R	SIM-1	80.00 ± 20.00	16.00 ± 9.80	4.17 ± 0.87
			SIM-2	60.00 ± 18.97	32.00 ± 14.97	2.38 ± 0.50
S. corneliomulleri	PER-584	L	SIM-1	100.00 ± 0.00	93.75 ± 6.25	8.07 ± 1.84
			SIM-2	100.00 ± 0.00	80.00 ± 14.58	4.50 ± 0.64
		R	SIM-1	100.00 ± 0.00	20.00 ± 10.95	4.40 ± 0.81
			SIM-2	64.00 ± 14.70	24.00 ± 7.48	1.67 ± 0.21
	PER-586	L	SIM-1	100.00 ± 0.00	81.25 ± 11.97	8.67 ± 2.22
			SIM-2	100.00 ± 0.00	75.00 ± 15.81	6.87 ± 1.04
		R	SIM-1	68.00 ± 18.55	12.00 ± 8.00	1.67 ± 0.33
			SIM-2	68.00 ± 20.59	20.00 ± 10.95	8.40 ± 2.25
	PER-592	L	SIM-1	100.00 ± 0.00	75.00 ± 0.00	6.47 ± 1.56
			SIM-2	100.00 ± 0.00	80.00 ± 9.35	6.69 ± 1.10
		R	SIM-1	100.00 ± 0.00	40.00 ± 20.98	4.00 ± 1.16
			SIM-2	84.00 ± 16.00	68.00 ± 18.55	3.44 ± 0.35
	T-040	L	SIM-1	100.00 ± 0.00	68.75 ± 11.97	2.18 ± 0.46
			SIM-2	100.00 ± 0.00	70.00 ± 20.00	3.50 ± 0.67
		R	SIM-1	64.00 ± 19.39	16.00 ± 7.48	1.50 ± 0.50
			SIM-2	48.00 ± 20.59	8.00 ± 4.90	2.00 ± 1.00
S. arcanum	ECU 777	L	SIM-1	92.00 ± 8.00	84.00 ± 7.48	5.11 ± 0.86
			SIM-2	80.00 ± 20.00	58.75 ± 21.25	5.73 ± 1.05
		R	SIM-1	68.00 ± 20.59	44.00 ± 18.33	2.09 ± 0.44
			SIM-2	20.00 ± 8.94	8.00 ± 4.90	1.50 ± 0.50
	ECU-766	L	SIM-1	100.00 ± 0.00	80.00 ± 11.55	3.56 ± 0.71
			SIM-2	100.00 ± 0.00	85.00 ± 9.57	8.82 ± 2.08
		R	SIM-1	48.00 ± 22.45	24.00 ± 16.00	3.67 ± 1.20
			SIM-2	36.00 ± 16.00	12.00 ± 4.89	6.00 ± 1.00

Table 5. (continued).

	ECU-775	L	SIM-1	95.00 ± 5.00	65.00 ± 12.58	2.77 ± 0.59
			SIM-2	76.00 ± 7.48	8.00 ± 8.00	6.00 ± 0.00
		R	SIM-1	75.00 ± 11.25	25.00 ± 18.93	1.80 ± 0.37
			SIM-2	44.00 ± 16.00	20.00 ± 20.00	1.60 ± 0.60
	ECU-783	L	SIM-1	100.00 ± 0.00	100.00 ± 0.00	3.90 ± 0.59
			SIM-2	76.00 ± 19.39	36.00 ± 19.39	3.33 ± 1.04
		R	SIM-1	88.00 ± 8.00	33.00 ± 9.95	1.88 ± 0.40
			SIM-2	52.00 ± 18.55	8.00 ± 4.90	3.50 ± 2.50
	LA-2185	L	SIM-1	60.00 ± 8.94	10.00 ± 5.77	1.00 ± 0.00
			SIM-2	36.00 ± 13.27	4.00 ± 4.00	2.00 ± 0.00
		R	SIM-1	24.00 ± 19.39	12.00 ± 12.00	1.33 ± 0.33
			SIM-2	4.00 ± 4.00	0.00 ± 0.00	0.00 ± 0.00
S. peruvianum	CH-20	L	SIM-1	100.00 ± 0.00	50.00 ± 17.32	3.60 ± 0.93
			SIM-2	100.00 ± 0.00	75.00 ± 19.36	2.07 ± 0.46
		R	SIM-1	76.00 ± 14.70	24.00 ± 11.66	2.00 ± 0.45
			SIM-2	52.00 ± 20.59	4.00 ± 4.00	1.00 ± 0.00
	ECU-106	L	SIM-1	65.00 ± 12.58	32.50 ± 11.09	2.00 ± 0.45
			SIM-2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		R	SIM-1	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.00
			SIM-2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	PER-412	L	SIM-1	100.00 ± 0.00	88.00 ± 8.00	6.14 ± 1.45
			SIM-2	100.00 ± 0.00	93.75 ± 6.25	7.56 ± 1.19
		R	SIM-1	88.00 ± 12.00	88.00 ± 12.00	3.86 ± 0.70
			SIM-2	100.00 ± 0.00	60.00 ± 18.26	2.83 ± 0.46

2001), in our assays leaf explants had higher regeneration capacity (B, R, PR, and Y) than roots.

High regeneration yield was obtained in *S. huaylasense* (3.86) and *S. corneliomulleri* (3.03) as is reported for other wild tomato species like *S. pennellii* (Trujillo-Moya et al., 2011; Trujillo-Moya and Gisbert, 2012) and *S. cheesmanii* (Arrillaga et al., 2001). However, lower values were obtained in *S. arcanum* (1.67) and *S. peruvianum* (1.35; Table 4). Productivity rate values in *S. peruvianum* s.str. (2.46) were similar to the values reported by Steinitz et al. (2006) and lower than those obtained by Peres et al. (2001) in *S. peruvianum* var. *humifusum* (currently *S. arcanum*). Although high regeneration ability was described in the *S. peruvianum* s.l. complex, where the high regeneration gene *Rg-1* was found (Koornneef et al., 1993), we classified as recalcitrant 2 of 3 *S. peruvianum* s.str. accessions: ECU-106 and CH-20. In all the species, the percentage of explants with buds was higher than the percentage of explants with shoots, which indicates that not all the buds are able to develop onto plants. Lack of bud development is commonly observed in explants of tomato and wild related species (Pratta et al., 1997; Steinitz et al., 2006).

More similar regeneration behavior was observed in *S. arcanum* accessions collected in a proximate area (ECU-777, ECU-766, ECU-775, and ECU-783) compared with the accession LA-2185 collected in another region (Figure 1; Table 1). Similar results were also observed in *S. corneliomulleri* (PER-586 and PER-584). However, in *S. huaylasense*, accessions collected in the same region (PE-18, PE-19, and PE-20) showed differences in terms of regeneration.

Correlations among leaf parameters (LN, LA, LD) showed a moderate correlation between LD and NL. For regeneration traits (B, R, PR, Y), a correlation between B and R was expected as buds are needed to develop plants. However, no relationship was obtained when comparing B and PR as not all the buds develop into shoots. High correlations were obtained among R, PR, and Y. This indicated that, in these accessions, the regeneration response is more uniform because a high number of explants with buds develop into plants. Finally, as leaf traits can be related to physiological characteristics (Kumar et al., 2012) we studied correlations between leaf and regeneration parameters. The results indicate

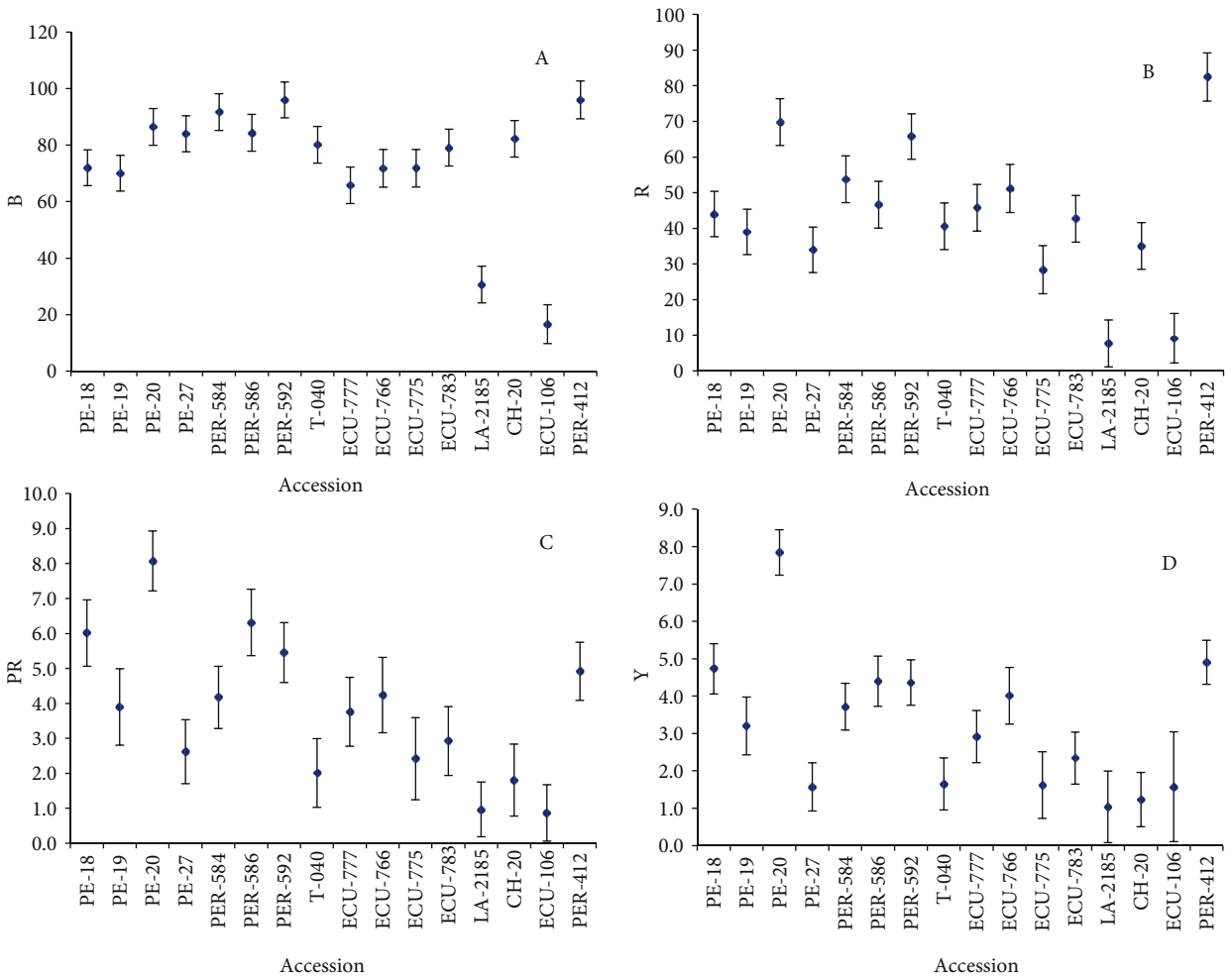

Figure 4. Means (±S.E.) for regenerating traits in accessions of *Solanum huaylasense*, *S. corneliomulleri*, *S. arcanum*, and *S. peruvianum* (s.str.). A- bud percentage (B), B- regeneration percentage (R), C- productivity rate (PR), and D- yield (Y).

Table 6. Correlation coefficients between residuals estimated for bud percentage (B), regeneration percentage (R), productivity rate (PR), and yield (Y) from in vitro cultured explants of *S. peruvianum* s.l. complex.

	R	PR	Y
B	0.67*	0.14	0.25*
R		0.40*	0.62*
PR			0.92*

Different from zero *P < 0.05.

Table 7. Correlation coefficients between residuals estimated for leaf morphology (number of leaflets (LN), leaflet margin dentation (LD), and leaflet area (LA)) and organogenesis response (bud percentage (B); regeneration percentage (R); productivity rate (PR) and yield (Y)).

	B	R	PR	Y
LN	0.47*	0.43*	0.53*	0.47*
LA	−0.36*	−0.35*	−0.32*	−0.29*
LD	0.51*	0.46*	0.37*	0.34*

Different from zero *P < 0.05.

that explants from leaves with high amounts of leaflets had high regeneration rates. A similar relationship was observed with LD and regeneration traits, whereas leaves with high area had a low response. Cytokinin regulates flexible leaf patterning by dynamic interaction with additional hormones and transcription factors (Shani et al., 2010). As the organogenic response may be influenced by endogenous hormones, our results could be related to a high endogenous cytokinin concentration in small and more dentate leaf explants.

In conclusion, we observed differences for leaf morphology and regeneration capacity among accessions of the 4 *Solanum* tested species; *S. arcanum*, *S. corneliomulleri*, *S. huaylasense*, and *S. peruvianum* s.str.. In plants cultured in vitro, the species can be differentiated by the number or the dentate shape of leaflets. However, leaf area only separated *S. arcanum* from the other species. Regarding regeneration, accession LA-2185 of *S. arcanum* and accessions ECU-106 and CH-20 of *S. peruvianum* s.str. can be considered low regenerating, whereas the remaining accessions had a good and high regeneration capacity. Although the 4 species are able to regenerate from root explants, we found leaf explants to be better for in vitro regeneration. Better results were also obtained in the organogenic medium SIM-1 with respect to SIM-2. Explants from leaves with a high amount and more dentate leaflets had higher regeneration.

Acknowledgments

C Trujillo thanks the Spanish 'Ministerio de Educación y Ciencia' for a predoctoral fellowship. This work has been funded by Universitat Politécnica de València (PAID 05 - 10). The authors acknowledge the germplasm bank of the Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV) for supplying accessions.

References

Alvarez AE, van de Wiel CCM, Smulders MJM, Vosman B (2001). Use of microsatellites to evaluate genetic diversity and species relationships in the genus *Lycopersicon*. Theor Appl Genet 103: 1283–1292.

Arrillaga I, Gisbert C, Sales E, Roig L, Moreno V (2001). In vitro plant regeneration and gene transfer on the wild tomato *Lycopersicon chesmanii*. J Hortic Sci Biotech 76: 413–418.

Bhatia P, Ashwath N, Senaratna T, David M (2004). Tissue culture studies of tomato (*Lycopersicon esculentum*). Plant Cell Tiss Org 78: 1–21.

Bedinger PA, Chetelat R, McClure B, Moyle LC, Rose JKC, Stack S, van der Knaap E, Baek Y, Lopez-Casado G, Covey PA et al. (2011). Interspecific reproductive barriers in the tomato clade: opportunities to decipher mechanisms of reproductive isolation. Sex Plant Reprod 24: 171–187.

Cap GB, Roberts PA, Thomason IJ, Murashige T (1991). Embryo culture of *Lycopersicon esculentum* × *L. peruvianum* hybrid genotypes possessing heat-stable resistance to *Meloidogyne incognita*. J Amer Soc Hort Sci 116: 1082–1088.

Chaerani R, Smulders MJ, van der Linden CG, Vosman B, Stam P, Voorrips RE (2007). QTL identification for early blight resistance (*Alternaria solani*) in a *Solanum lycopersicum* × *S. arcanum* cross. Theor Appl Genet 114: 439–450.

Chen LZ, Adachi T (1998). Protoplast fusion between *Lycopersicon esculentum* and *L. peruvianum*-complex: somatic embryogenesis, plant regeneration and morphology. Plant Cell Rep 17: 508–514.

Devi R, Dhaliwal MS, Kaur A, Gosal SS (2008). Effect of growth regulators on in vitro morphogenic response of tomato. Indian J Biotechnol 7: 526–530.

Faria RT, Destro D, Bespalhok JC, Illg RD (2002). Introgression of in vitro regeneration capability of *Lycopersicon pimpinellifolium* Mill. into recalcitrant tomato cultivars. Euphytica 124: 59–63.

Foolad MR, Merk HL, Ashrafi H (2007). Genetics, genomics and breeding of late blight disease and early blight disease in tomato. Crit Rev Plant Sci 27: 75–107.

Gisbert C, Arrillaga I, Roig LA, Moreno V (1999). Acquisition of a collection of *Lycopersicon pennellii* (Corr. D'Arcy) transgenic plants with *uid*A and *npt*II marker genes. J Hortic Sci Biotech 74: 105–109.

Jablonska B, Ammiraju JSS, Bhattarai K, Mantelin S, Martinez de Ilarduya O, Roberts PA, Kaloshian I (2007). The *Mi- 9* Gene from *Solanum arcanum* conferring heat-stable resistance to root-knot nematodes is a homolog of *Mi-* 1. Plant Physiol 143: 1044–1054.

Jiang W, Barshan Özaktaş B, Mantri N, Tao Z, Lu H (2013). Classification of *Camellia* species from 3 sections using leaf anatomical data with back-propagation neural networks and support vector machines. Turk J Bot 37: 1093–1103.

Khoudi H, Nouri-Khemakhem A, Gouiaa S, Masmoudi K (2009). Optimization of regeneration and transformation parameters in tomato and improvement of its salinity and drought tolerance. Afr J Biotechnol 8: 6068–6076.

Kochevenko A, Ratushnyak Y, Korneyeyev D, Stasik O, Porublyova L, Kochubey S, Suprunova T, Gleba Y (2000). Functional cybrid plants of *Lycopersicon peruvianum* var 'dentatum' with chloroplasts of *Lycopersicon esculentum*. Plant Cell Rep 19: 588–597.

Koornneef M, Hanhart CJ, Martinelli L (1987). A genetic analysis of cell culture traits in tomato. Theor Appl Genet 74: 633–641.

Koornneef M, Bade J, Hanhart C, Horsman K, Schel J, Soppe W, Vekerk R, Zabel P (1993). Characterization and mapping of a gene controlling shoot regeneration in tomato. Plant J 3: 131–141.

Kumar V, Kodandaramaiah J, Vati Rajan M (2012). Anatomical traits in relation to physiological characteristics in mulberry (*Morus* sp.) cultivars. Turk J Bot 36: 683–689.

Kut SA, Evans DA (1982). Plant regeneration from cultured leaf explants of eight wild tomato species and two related Solanum species. In Vitro Cell Dev B 8: 593–598.

Marchionni BE, Pratta GR, Zorzoli R (2007). Genetic analysis of the in vitro culture response in tomato. Plant Cell Tiss Organ 88: 233–239.

Miller JC, Tanksley SD (1990). RFLP analysis of phylogenetic relationships and genetic variation in the genus Lycopersicon. Theor Appl Genet 80: 437–448.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15: 473–497.

Parker-Norton J, Boll WG (1954). Callus and shoot formation from tomato roots in vitro. Science 119: 220–221.

Peralta IE, Knapp S, Spooner DM (2005). New species of wild tomatoes (*Solanum* section *Lycopersicon*: Solanaceae) from Northern Peru. Syst Bot 30: 424–434.

Peralta IE, Spooner DM, Knapp S (2008). Taxonomy of wild tomatoes and their relatives (*Solanum* sections *Lycopersicoides*, *Juglandifolia*, *Lycopersicon*; Solanaceae). Syst Bot M 84: 1–186.

Pereira-Carvalho RC, Boiteux LS, Fonseca MEN, Díaz-Pendón JA, Moriones E, Fernández-Muñoz R, Charchar JM, Resende RO (2010). Multiple resistance to *Meloidogyne* spp. and to bipartite and monopartite *Begomovirus* spp. in wild *Solanum* (*Lycopersicon*) accessions. Plant Dis 94: 179–185.

Peres LEP, Morgante PG, Vecchi C, Kraus JE, Sluys MA (2001). Shoot regeneration capacity from roots and transgenic hairy roots of tomato cultivars and wild related species. Plant Cell Tiss Org 65: 37–44.

Pratta G, Zorzoli R, Picardi LA (1997). Intra and interspecific variability of in vitro culture response in *Lycopersicon* tomatoes. Braz J Genet 20: 75–78.

Ruf S, Hermann M, Berfr IJ, Carrer H, Bock R (2001). Stable genetic transformation of tomato plastids and expression of foreign protein in fruit. Nature Biotechnol 19: 870–875.

Sacks EJ, Gerhardt LM, Gtaham EB, Jacobs J, Thorrup TA, Clair DA (1997). Variation among 41 genotypes of tomato (*Lycopersicon esculentum* Mill.) for crossability to *L. peruvianum* (L.) Mill. Ann Bot-London 80: 469–477.

Satoh H, Takashina T, Escalante A, Egashira H, Imanishi S (2000). Molecular markers mapped around the high shoot regeneration capacity gene *Rg-2* in *Lycopersicon chilense*. Breeding Sci 50: 251–256.

Segeren MI, Sondahl MR, Siqueira WJ, Medina Filho HP, Nagai H, Lourencao AI (1993). Tomato breeding: 1. Embryo rescue of interspecific hybrids between *Lycopersicon esculentum* Mill. and *L. peruvianum* Mill. Braz J Genet 16: 367–380.

Shani E, Ben-Gera H, Shleizer-Burko S, Burko Y, Weiss D, Ori N (2010). Cytokinin regulates compound leaf development in tomato. Plant Cell 22: 3206–3217.

Smith PG (1944). Embryo culture of a tomato species hybrid. P Am Soc Hortic Sci 44: 413–416.

Spooner DM, Peralta IE, Knapp S (2005). Comparison of AFLPs with other markers for phylogenetic inference in wild tomatoes [Solanum L. section Lycopersicon (Mill.) Wettst.]. Taxon 54: 43–61.

Steinitz B, Amitay A, Gaba V, Tabib Y, Keller M, Levin I (2006). A simple plant regeneration ability assay in a range of *Lycopersicon* species. Plant Cell Tiss Org 84: 269–278.

Stommel JR, Sinden SL (1991). Genotypic differences in shoot forming capacity of cultured leaf explants of *Lycopersicon hirsutum*. HortScience 26: 1317–1320.

Takashina T, Suzuki T, Egashira H, Imanishi S (1998). New molecular markers linked with the high shoot regeneration capacity of the wild tomato species *Lycopersicon chilense*. Breeding Sci 48: 109–113.

Thomas BR, Pratt D (1981). Efficient hybridization between *Lycopersicon esculentum* and *L. peruvianum* via embryo cells. Theor Appl Genet 59: 215–219.

Trujillo-Moya C, Gisbert C, Vilanova S, Nuez F (2011). Localization of QTLs for in vitro plant regeneration in tomato. BMC Plant Biol 11: 140.

Trujillo-Moya C, Gisbert C (2012). The influence of ethylene and ethylene modulators on shoot organogenesis in tomato. Plant Cell Tiss Org 111: 41–48.

Zuriaga E, Blanca J, Nuez F (2009). Classification and phylogenetic relationships in *Solanum* section *Lycopersicon* based on AFLP and two nuclear gene sequences. Genet Resour Crop Ev 56: 663–678.