# *Comparative androgenetic competence of various species and genotypes within the genus* Pisum *L.*

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**Plant Cell, Tissue and Organ Culture (PCTOC)**
Journal of Plant Biotechnology

ISSN 0167-6857

Plant Cell Tiss Organ Cult
DOI 10.1007/s11240-020-01934-y

Springer

Plant Cell, Tissue and Organ Culture (PCTOC)
https://doi.org/10.1007/s11240-020-01934-y

ORIGINAL ARTICLE

# Comparative androgenetic competence of various species and genotypes within the genus *Pisum* L.

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Received: 23 May 2020 / Accepted: 14 September 2020


## Abstract

In pea breeding is important to shorten the generation cycles to obtain homozygosity quickly. Doubled haploid technology is important to attain this purpose and androgenesis is the most promising tool for induction of haploids in legumes. Commercial pea varieties have been described as recalcitrant to this approach but very little is known regarding the androgenic competence of pea relatives. In this work, a comparative study of the androgenic response among different taxa of the genus *Pisum* was undertaken. We cultured anthers of 11 pea materials from the primary and secondary genepools under the same experimental conditions, and studied their competence to produce calli and plants in vitro. Significant differences were found in the percentage of callus and plant production between the different species and subspecies. The two wild forms *Pisum fulvum* Sibth. & Sm. and *Pisum sativum* subsp. *elatius* (Bieb.) Aschers. & Graebn. regenerated shoots from anther culture with the highest efficiency (67% and 38%, respectively), becoming potential sources of androgenic competence. Among the cultivated genotypes of *P. sativum*, the botanical variety *arvense* regenerated shoots with the highest percentage (40%) also being a good candidate to study androgenesis. The commercial varieties tested showed significant differences in the callus and plant production, with Primogénita (FCA-INTA) and B101 giving the best results although with low plant regeneration percentages (17% and 11%, respectively). *P. fulvum*, *P. sativum* subsp. *elatius* and *P. sativum* subsp *sativum* var *arvense* were identified as highly responsive to anther culture, useful to transfer androgenesis competence to recalcitrant commercial varieties.

## Key message

Within genus *Pisum*, the wild forms *P. fulvum*, *P. sativum* subspecies *elatius* and the cultivated variety *arvense* were identified as potential sources to introduce androgenesis competence into recalcitrant commercial varieties.

**Keywords** *Pisum* · Anther culture · Wild relatives · Androgenic response · Plant regeneration

## Introduction

The *Pisum* genus includes wild species as *P. fulvum* found in the middle east (Smýkal et al. 2017), the cultivated species *P. abyssinicum* from Yemen and Ethiopia, which was likely domesticated independently of *P. sativum*, and a large and loose aggregate of both wild (*P. sativum* subsp. *elatius*) and cultivated forms that comprise the species *P. sativum* in a broad sense (Trněný et al. 2018) which is native to the Europe–Mediterranean region and middle and northwest Asia (Smýkal et al. 2017). Two gene pools were found in this genus. The primary gene pool includes *P. sativum* with its different subspecies, botanical and commercial varieties meanwhile the secondary gene pool is composed of *P.*

Communicated by Sergio J. Ochatt.

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Published online: 21 September 2020

*fulvum* and *P. abyssinicum* (Coyne et al. 2020). Here we adopt the taxonomic system of *Pisum* outlined by Maxted and Ambrose (2001) and Bogdanova et al. (2020). According to this generalized system, the genus embraces three species namely, *P. sativum* L., *subsp. sativum* (includes var. *sativum* and var. *arvense*); *subsp. elatius* (Bieb.) Aschers. & Graebn (includes var. *elatius*, var. *brevipedunculatum* and var. *pumilio*), *P. fulvum* Sibth. & Sm.; *P. abyssinicum* A. Br.

Pea is a self-pollinated diploid (2n = 14, x = 7) annual crop. Modern breeding programs typically use bulk populations, pedigree selection or SSD methodology to obtain new cultivars through hybridizations between commercial varieties. However, the genetic variation for improvement of several economically important traits is inadequate at best and, in many cases, absent from available germplasm (Smýkal et al. 2018a; Coyne et al. 2020). Therefore, natural resistance must be introduced by hybridization with wild related species. These traits identified in *P. fulvum* include insect resistance (Esen et al. 2019), improved seed composition and resistance to several soil-borne fungal pathogens (Barilli et al. 2018), drought tolerance (Naim-Feil et al. 2017; Kosterin et al. 2020), besides seed yield related traits (Mikíc et al. 2013). Several authors capitalized on *P. fulvum* cross-compatibility with *P. sativum* to produce weevil-resistant interspecific progeny (Byrne et al. 2008; Clemente et al. 2015) and hybrids with improved tolerance to *Aphanomyces euteiches* root rot using *P. sativum* as the female and *P. fulvum* as the male parent (Ochatt et al. 2004). Bobkov and Selikhova (2017) created *P. sativum* introgression lines with inclusions of new genes and alleles of economically valuable traits from the *P. fulvum* genome. As *P. fulvum*, wild peas (*P. sativum* subsp. *elatius*) are practically important as a source of genetic diversity potentially valuable for pea breeding, first of all genes for resistance to various pests, diseases and draught (Kosterin, 2016). Porter (2010) identified tolerance to *Fusarium* root rot in *P. sativum* subsp. *elatius* germplasm with high levels of partial resistance. Further, Mikíc et al. (2013) and Clemente et al. (2015) identified wild pea accessions (*P. sativum* subsp. *elatius*) with pronounced reduced protease inhibitor activity in seeds.

The use of wide crosses to source key traits results in breeding difficulties as wild-type traits are introduced and crop productivity requires many years to be restored by backcrosses.

In pea, shortening the breeding cycle is important to obtain homozygosity quickly, and doubled haploidy is an important technology to attain this purpose. Different methodologies can be used to obtain haploid plants such as wide hybridization with chromosome elimination, gynogenesis and androgenesis (anther and microspore culture) depending on the species (Khush and Virmani 1996). Among these techniques, androgenesis seems to be most promising for induction of haploids in legumes (Gatti et al. 2016). Nevertheless, there have been very few reports of haploid plant production in pea, which has been described as recalcitrant to this approach (Gupta 1975; Croser and Lülsdorf 2004; Sidhu and Davies 2005; Ochatt et al. 2009; Bobkov 2010, 2014; Lulsdorf et al. 2011; Ribalta et al. 2012) because the regeneration frequencies of complete haploid plants were low (Ochatt et al. 2009; Bobkov 2014). All these reports used only commercial pea varieties, or mutants from them, and nearly nothing is known regarding the androgenic competence of pea relatives. Doubled haploids also represent a useful tool for genetic analyses of traits of interest for plant breeding or related to domestication (Salas et al. 2011). However, to our knowledge, a comparative study of the androgenic response of different taxa of the genus *Pisum*, is still lacking. The objective of this work was to compare and analyze in vitro responses to the anther culture among different taxa of the genus *Pisum* in order to identify androgenesis-responsive materials potentially useful as model systems or as potential sources of androgenic competence.

## Materials and methods

### Plant material

Eleven pea genotypes belonging to different species, subspecies, botanical varieties and commercial varieties of the genus *Pisum* were used. These accessions have been additionally characterized at the morphological and molecular levels (Espósito et al. 2007; Gatti et al. 2011, 2017). With the collected morphological data, Euclidean distances between genotypes were calculated and a cluster analysis was carried out. A dendrogram was generated using the average linkage method through the InfoStat software (Di Rienzo et al. 2012) (Fig. 1). In this Figure the different used genotypes and their phylogenetic relationships are shown.

### Donor plant growth conditions

In 2018, seeds of the different pea genotypes were sown (June 15) and grown in the experimental field "J. F. Villarino" at the research station of the faculty of Agriculture of Rosario University, Argentina (331′ S and 6053′ W), under natural light conditions, following a randomized complete design with rows of 50 plants per genotype. Spacing between plants and rows was 0.10 and 0.70 m, respectively. Climate data were collected from the Pegasus meteorological station (Tecmes) located in the premises of the College of Agriculture, National University of Rosario, Villarino, Zavalla, Santa Fe.

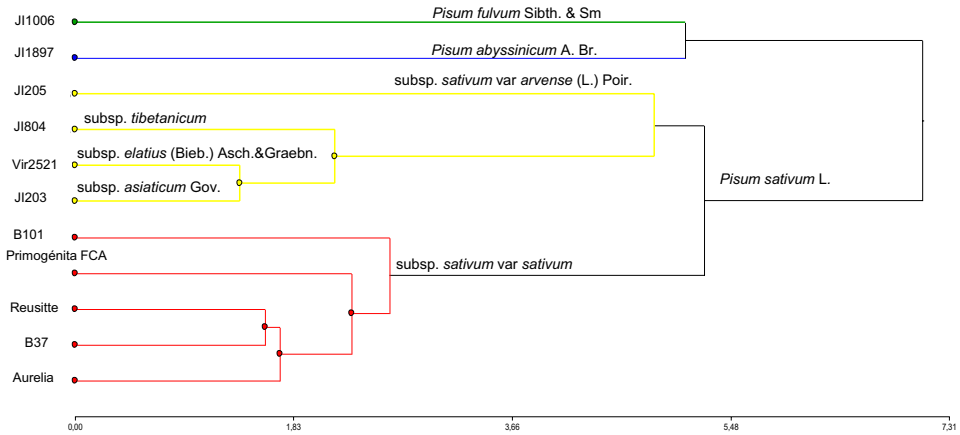


**Fig. 1** Dendrogram compiled by Average linkage method showing the grouping of 11 pea genotypes used in this work belonging to different taxa within genus *Pisum* based on morphological traits (Euclidean distances)

## Anther culture

Flower buds were extracted from the donor plants when they reached an approximate length of 6–7 mm corresponding to the uninucleated state (Croser et al. 2006; Ochatt et al. 2009). The flower buds underwent two treatments, the control treatment without cold pretreatment and a cold pretreatment at 4 °C for 3 days (Ochatt et al. 2009). They were then sterilized in 70% alcohol for 1 min, 25% sodium hypochlorite for 10 min and 3 rinses with sterile distilled water. The anthers were excised and cultured in vitro onto callus induction media containing MS (Murashige and Skoog 1962) formula, 6% sucrose and 0.6% agar–agar with different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) (0, 0.5 and 1 mg/L, Medium 1, 2 and 3, respectively) and pH 5.5. A total of 150 anthers per genotype and medium were kept for 1 month in a culture room at a 16:8 h light:dark regime with a photosynthetic photon flux density of 30 µmol $m^{-2}$ $s^{-1}$ from cool white fluorescent lamps, and at 25 ± 1 °C. Calli that exhibited embryo formation were transferred to a solid embryo maturation medium containing MS formula, 3% sucrose, 0.6% agar, 1 mg/L α-naphthaleneacetic acid (NAA) and 0.5 mg/L gibberellic acid ($GA_3$). Finally, mature embryos (cotyledonary phase) were transferred to plant regeneration medium containing ½ salt strength MS, with 4% sucrose, 0.6% agar, 5 mg/L 6-benzylaminopurine (BAP), 0.25 mg/L IAA (indole-3yl-acetic acid) and 0.25 mg/L NAA and 1 mg/L $GA_3$. For all media, the pH was adjusted to 5.5 prior to autoclaving. Cultures were maintained in a growth chamber in the same conditions as indicated above.

## Acclimatization

After rooting, plantlets were removed from culture tubes and glass vials, washed thoroughly with distilled water to remove the remaining medium and planted in plastic pots containing sterile In Vitro Soil-less (IVS) medium for acclimatization. IVS medium comprised of sphagnum peat, coarse river sand (1–3 mm diameter), and perlite (Horticulture grade P500) at a ratio of 0.5:2:2 was sterilized for 40 min at 121 °C prior to use (Bermejo et al. 2012). For the first month, plantlets were covered with transparent polyethylene bags to maintain high humidity. They were watered once a week using half-strength MS salt solution. When the bags were removed plantlets were watered twice a week with distilled water. The plants were kept in a growth room at 23 ± 2 °C and a 16:8 h light regime (30 µmol $m^{-2}$ $s^{-1}$ from cool white fluorescent lamps).

## Data analysis

The experiment was repeated twice. The effects of culture media and pretreatment on callus induction were evaluated and since the variables did not follow a normal distribution, a Kruskal–Wallis test was performed using the Infogen program (Balzarini and Di Rienzo 2003). The effect of genotype on the percentage of callus production and plant regeneration was also evaluated and subjected to analysis of variance (ANOVA) using a randomized complete design. The means were separated at P = 0.05 level of significance according to Tukey's test using Infogen software (Balzarini and Di Rienzo 2003). Data that did not have a normal distribution in residual plot analysis were transformed, prior to ANOVA, through square root (x + 0.5).

# Results

## Abiotic stresses of donor plants

The field-grown donor plants were subjected to multiple environmental stresses during the period of seedling development until the harvest of flower buds (June 15–September 23). During this period, rainfall amounts, and average temperatures were variable. Rainfall amounted to 11.5 mm in this period reaching 5 mm in the months of June and July,

and only 1.25 mm in September with no rains in August thus being considered a period of drought. The average temperatures were 14 ºC in June, 10 ºC in July with periods of freezing temperatures (0 ºC), 17 ºC in August and 25 ºC in September. Strong winds with gusts of 30 km/h were also observed sporadically during this period.

## Anther culture

Extracted flower buds were around 7 mm long, with 1-mm-long anthers, and which were translucent and light yellow (Fig. 2a). Differences in optimum flower bud size within the genus existed, but were minimal.

The percentage of callus formation was significantly higher for flower buds without cold pretreatment compared with pretreatment at 4 °C ($H = 11.25$, $p < 0.001$, Table 1). As it is shown in Table 1, all the genotypes showed superior behavior when flower buds did not receive a cold pretreatment. With regard to the effect of different 2,4-D concentrations on the callus percentage, MS medium without hormones (Medium 1) was not efficient for callus formation; only one cultivar from *P. sativum* subsp *sativum* (Aurelia) gave a low callus percentage (3%) on this medium (Table 1). Green embriogenic calli were produced from anthers of the different genotypes on culture media containing 2,4-D (Fig. 2b), with Medium 3 significantly superior ($H = 5.96$; $p < 0.05$, Table 1). Although there were genotypes such as *Pisum sativum* subsp. *elatius* Vir2521 and the commercial variety Aurelia of *Pisum sativum* subsp. *sativum* that showed higher callus percentages without cold pretreatment in the Medium 2 (containing an intermediate concentration of 2,4-D), there were also genotypes that could not induce callus in this medium (JI203, JI804, B37, Table 1). In contrast, in Medium 3 containing the highest 2,4-D concentration, all genotypes were able to induce callus formation without cold pretreatment. When comparing all the genotypes, *Pisum abyssinicum* and the commercial variety B101 gave the greatest number of calli on the Medium 3 and without a flower buds pretreatment (Table 1). Thus, the different *Pisum* taxa were tested using the best conditions for anther culture ie. without cold pretreatment and on Medium 3 for callus induction.

Embryogenesis proceeded through the typical successive stages (i.e. globular, heart, torpedo and cotyledonary) of somatic embryo induction, expression, growth and ultimate conversion to plants (Fig. 2c, d, e, f, g).

Comparing the different species, variance analysis showed significant effects on the percentage of callus induction ($F = 24.5$; $p < 0.001$) and plant regeneration ($F = 27.9$; $p < 0.001$). Although anthers of the cultivated

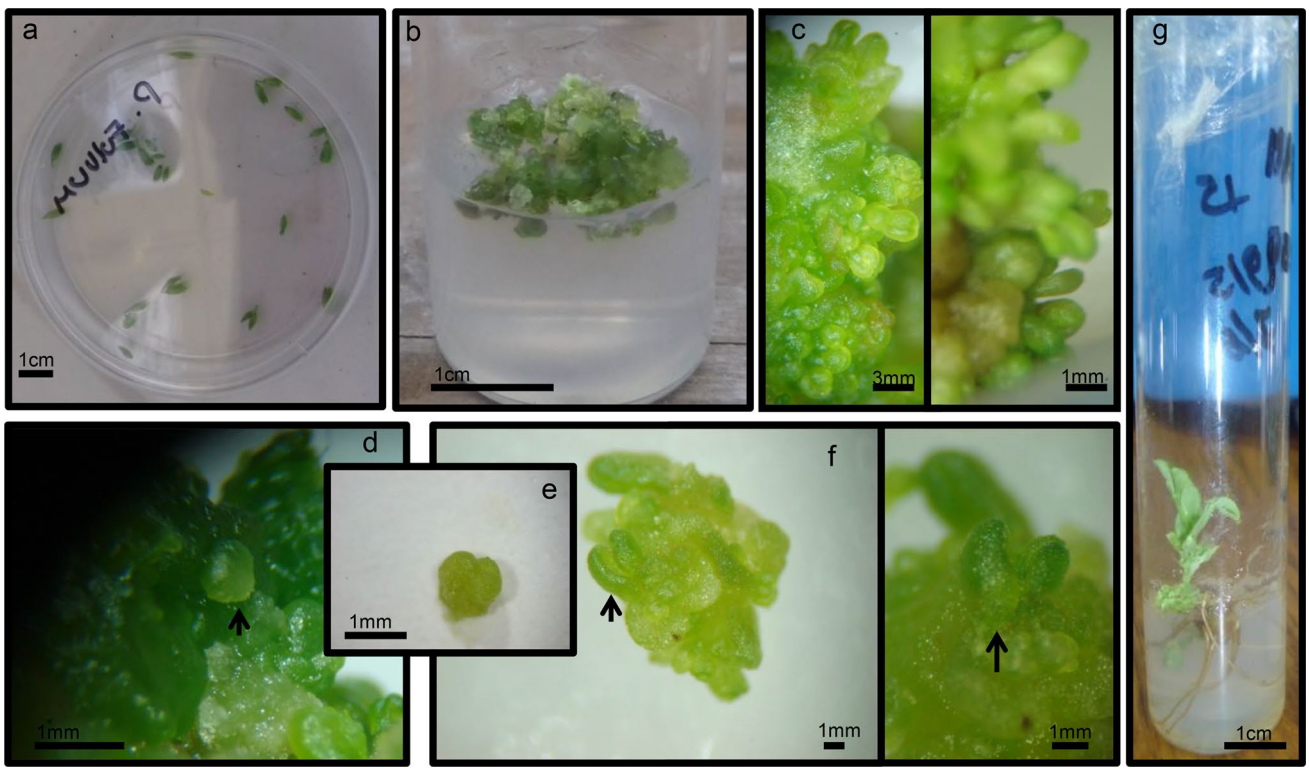


**Fig. 2** The in vitro anther culture protocol. **a** Flower buds of *P. fulvum* in the uninucleated state **b** Formation of green embryogenic callus in Medium 3. **c** Callus showing different stages of somatic embryogenesis on embryo maturation medium **d** globular, **e** heart shaped **f** cotyledonary phase, indicated by arrows **g** Pea plantlet development in plant regeneration medium

**Table 1** Effect of flower buds cold pretreatment at 4 °C for 3 days, culture media containing different concentrations of 2,4-D (0, 0.5 and 1 mg/L, Medium 1, 2 and 3, respectively) and 11 *Pisum* L. genotypes on the percentage of anthers producing calli (Callus percentage)

| Genotypes | Callus percentage (%) | | | | | |
|---|---|---|---|---|---|---|
| | Medium 1 | | Medium 2 | | Medium 3 | |
| | without cold[a] | 4 ºC for 3days | without cold | 4 ºC for 3days | without cold | 4 ºC for 3days |
| JI1006 | 0.00 b | 0.00 a | 14.29 b | 6.00 a | 18.75 b | 0.00 c |
| JI1897 | 0.00 b | 0.00 a | 10.64 c | 0.00 c | 38.90 a | 4.40 b |
| JI203 | 0.00 b | 0.00 a | 0.00 f | 0.00 c | 2.13 e | 0.00 c |
| Vir2521 | 0.00 b | 0.00 a | 33.33 a | 2.38 b | 2.22 e | 0.00 c |
| JI804 | 0.00 b | 0.00 a | 0.00 f | 0.00 c | 3.77 de | 0.00 c |
| JI205 | 0.00 b | 0.00 a | 8.00 cd | 2.78 b | 20.00 b | 0.00 c |
| B37 | 0.00 b | 0.00 a | 0.00 f | 0.00 c | 6.56 cd | 0.00 c |
| B101 | 0.00 b | 0.00 a | 10.20 c | 0.00 c | 39.00 a | 3.40 b |
| Primogénita FCA | 0.00 b | 0.00 a | 14.89 b | 7.30 a | 16.00 b | 14.80 a |
| Aurelia | 3.00 a | 0.00 a | 6.25 d | 0.00 c | 3.70 de | 0.00 c |
| Reusitte | 0.00 b | 0.00 a | 2.22 e | 2.13 b | 10.00 c | 5.00 b |

| Pretreatment | N | Median | Ranks[b] | H | p |
|---|---|---|---|---|---|
| without cold | 66 | 2.22 | 74.92 a | 11.25 | 0.00057 |
| 4 ºC for 3 days | 66 | 0.00 | 58.08 b | | |
| Medium | | | | | |
| 1 | 44 | 0.00 | 38.73 c | 5.96 | 0.01 |
| 2 | 44 | 2.30 | 73.95 b | | |
| 3 | 44 | 4.68 | 86.82 a | | |

[a]Mean values

[b]Ranks of 2 replicates with the same letter within a column are not significantly different at $p \leq 0.05$ according to Kruskal–Wallis non-parametric variance analysis test

JI1006 *Pisum fulvum* Sibth. & Sm., JI1897 *Pisum abyssinicum* A. Br., JI203 *Pisum sativum* subsp. *asiaticum* Gov., Vir2521 *Pisum sativum* subsp. *elatius* (Bieb.) Aschers. & Graebn., JI804 *Pisum sativum* subsp. *tibetanicum*, JI205 *Pisum sativum* subsp. *sativum* var *arvense* (L.) Poir., B37, B101, Primogénita FCA, Aurelia and Reusitte *Pisum sativum* L. subsp. *sativum* var *sativum*

species *P. abyssinicum* gave a greater number of calli, the wild species (*P. fulvum*) regenerated plants with a higher efficiency (67%) (Fig. 3a).

Significant differences were also found in the percentage of callus and plant production between the different subspecies analyzed ($F = 65.2$; $p < 0.001$ and $F = 15.4$; $p < 0.001$, respectively). Thus, *P sativum* subsp. *sativum* yielded the greatest percentage of callus induction and coupled with plant regeneration, while *P. sativum* subsp. *elatius* gave a lower callus induction but coupled with the highest plant regeneration percentage (38%) (Fig. 3b).

With regard to the botanical varieties, no significant differences were found in terms of the callus induction but the percentage of plant regeneration was significantly higher for *P sativum* subsp. *sativum* var. *arvense* (40%) ($F = 14.9$; $p < 0.01$) (Fig. 3c).

The different commercial varieties also showed significant differences in the percentage of callus induction ($F = 69.1$; $p < 0.05$) and plant regeneration ($F = 23.7$; $p < 0.05$), with the varieties Primogénita (FCA-INTA) and B101 exhibiting the best values (Fig. 3d).

Unfortunately, all regenerated plants were extremely enfeebled and did not survive green-house transfer so the ploidy level could not be determined.

## Discussion

Androgenesis is the most widely used in vitro method of haploid induction (Kasha 2005). The in vitro culture of the whole immature anthers, containing microspores, is the simplest method of haploid induction through the androgenetic pathway (Niazian and Shariatpanahi 2020) and is affected by many factors: genotype, donor plant growth conditions, microspore stage, pre-treatment of flower buds, culture media, type and concentration of growth regulators, among others (Sidhu and Davies 2005; Wang et al. 2018).

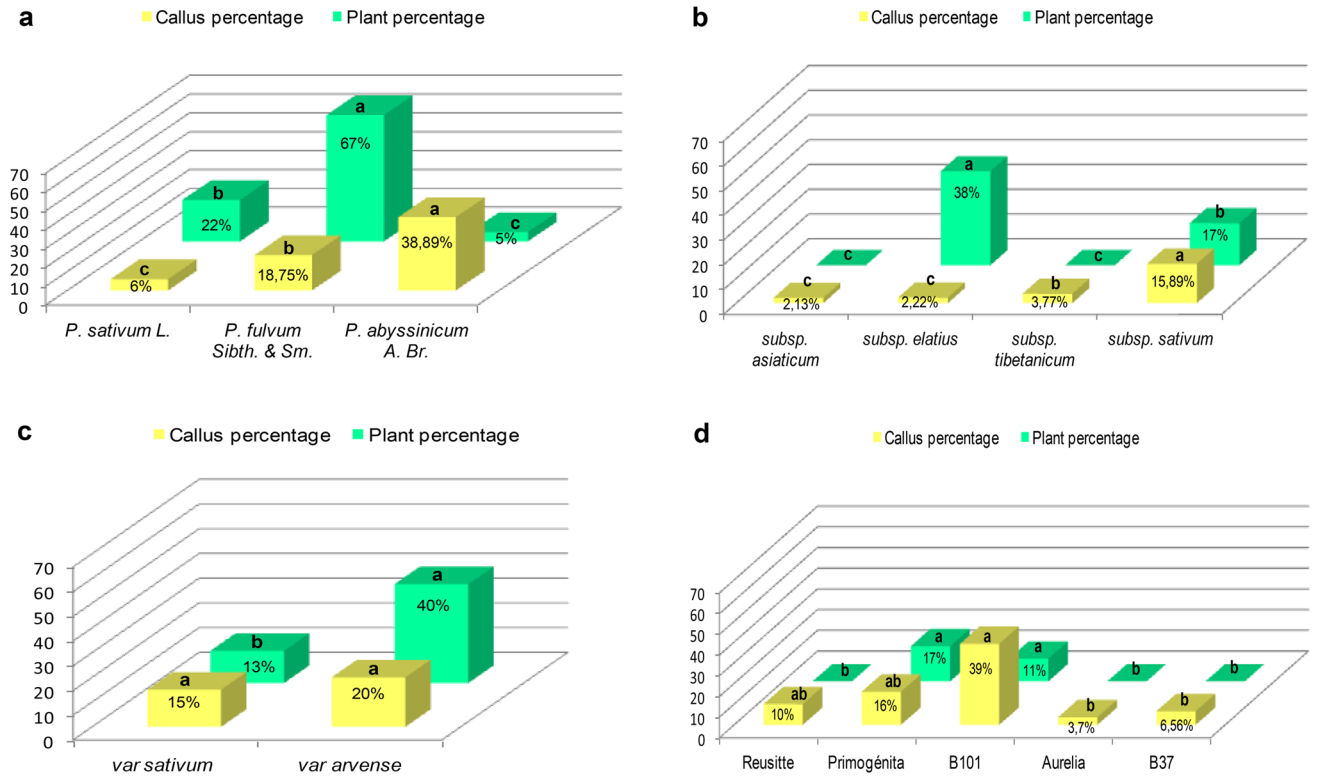


**Fig. 3** Effect of different taxa within *Pisum* L. on the percentage of anthers producing calli (Callus percentage) and the percentage of calli producing plants (Plant percentage) without cold pretreatment of flower buds and on Medium 3 for callus induction. Different letters indicate significant differences between genotypes (Tukey's test, $p < 0.05$). **a** Different species of *Pisum* L.: *Pisum fulvum* Sibth. & Sm. accession JI1006, *Pisum abyssinicum* A. Br. accession JI1897 and *Pisum sativum* L. represented by the mean value from accessions JI203, Vir2521, JI804, JI205, B37, B101, Primogénita FCA, Aurelia and Reusitte. **b** Different subspecies of *Pisum sativum*: subsp. *asiaticum* accession JI203, subsp. *elatius* accession Vir2521, subsp. *tibetanicum* accession JI804 and subsp. *sativum* represented by the mean value from accessions JI205, B37, B101, Primogénita FCA, Aurelia and Reusitte. **c** Different botanical varieties of *Pisum sativum* subspecies *sativum*: var *arvense* accession JI205 and var *sativum* represented by the mean value from accessions B37, B101, Primogénita FCA, Aurelia and Reusitte. **d** Different commercial varieties of *Pisum sativum* subspecies *sativum* var *sativum*: B37, B101, Primogénita FCA, Aurelia and Reusitte

Stress actions are used for reprogramming microspores to the sporophytic path of development. In anther and microspore culture the stress treatments influence the efficiency of androgenesis in plant species (Abdollahi and Rashidi 2018). Ochatt et al. (2009) obtained the best proliferation responses in pea with microspores isolated from anthers excised from flower buds that were stored at 4 ºC for at least 2 d in the dark before being sterilized. Bobkov (2010, 2014) also noted that treatment of pea buds with cold (4 °C) for more than 48 h increased the number of microcalli. Conversely, in our study, cold treatment of flower buds at 4 ºC for 3 days was not beneficial for pea androgenesis induction. For cowpea also no cold pre-treatment was necessary for androgenesis induction (Croser et al. 2006).

With regard to the donor plant growth conditions, in soybean, for example, field-grown plants are routinely used (Cardoso et al. 2004; Rodrigues et al. 2004; Deswal 2018) however, the pea literature to date use flower buds collected from mother plants grown under controlled conditions (greenhouse, growth chambers or phytotrons). In our study we used flower buds collected directly from field material, so the donor plants were subjected to multiple abiotic stresses induced by nature such as low temperatures, periods of rain and drought and strong winds during the period of seedling development until the harvest of flower buds. As the donor plants were subjected to this set of environmental stresses, perhaps the subsequent pretreatment of the flower buds to the experimental conditions of cold for 3 days did not have a significant effect in the androgenesis induction. Grewal et al. (2009) and Ochatt et al. (2009) showed that there was a positive effect of pyramiding stress factors on the induction of embryo formation from intact anthers. The application of combined stress factors seems to be the way to overcome recalcitrance of legumes to androgenesis, likely mediated through increases in hormone levels in stressed anthers (Lulsdorf et al. 2011). Even though these reports in pea utilized pretreatments of floral buds *ex-planta*, in soybean, Garda et al. (2020) used floral buds *in-planta* that involved

the whole donor plant and indicated that androgenesis can be stimulated up to 9–12% induction frequency by the use of pyramidal stressors (a combination of an extended cold 10 °C day/8 °C night shock *in-planta* for 3 days then overnight cold shock at 4 °C, a series of incubation temperatures from 11 °C to 18 °C to 25 °C, and nitrogen starvation medium). Ochatt et al. (2009) and Lüsdorf et al. (2011) also showed that high temperatures are detrimental to microspore viability but in contrast, cold storage was always beneficial, even for long periods of time without any detrimental effect on the subsequent viability of cultured anthers or the division competence of cultured microspores. Islam and Tuteja (2012) observed the highest percentage of androgenesis induction and plant regeneration in wheat when spikes were subjected to drought stress for 3 h. In the current study, since periods of very low temperatures and drought and no periods of high temperatures were detected during the growth of the donor plants, these stress conditions could have been beneficial for the induction of androgenesis. A greater androgenic response also may be related to metabolic changes induced by the donor plants' response to the cold shock as in the model *Brassica* system (Ferrie and Caswell 2011) and soybean system (Garda et al. 2020). In addition, since it was not need to perform pre-treatments in the laboratory, our protocol becomes a cheaper and simpler system, where mother plants can express their maximum potential, obtaining more vigorous plants, with the production of a large number of flower buds.

The choice of plant growth regulator and relative auxin:cytokinin ratio is an important feature of androgenesis induction media (Croser et al. 2006). Lulsdorf et al. (2011) showed that phytohormones especially auxins play a major role in induction of androgenesis in legumes. In many anther culture systems, 2,4-D, is considered as one of the most important auxins, because it elicits rapid cell proliferation and callus formation (Zheng and Konzak 1999). In our study, the medium with the highest 2,4-D concentration (Medium 3) was the most efficient to induce calli formation from the pea anthers. The positive effect of a high concentration of 2,4-D on the frequency of callus production has also been reported in *Cicer arietinum* L. anther culture by Abdollahi and Rashidi (2018).

Croser et al. (2006) and Lulsdorf et al. (2011) determined that the critical factor in the development of an efficient androgenesis protocol is the identification of most responsive genotypes. In our work we cultured anthers of 11 pea genotypes from the primary and secondary genepools under the same experimental conditions, and studied their competence to produce calli, shoots and roots. These genotypes constitute a good representation of the genetic variability found in the genus *Pisum* (Gatti et al. 2017).

With regard to the species and subspecies analyzed, *Pisum fulvum* is a wild species and *P. sativum* subsp. *elatius* is considered a wild line representative of the *P. sativum* species (Kosterin and Bogdanova 2015). In our study, although no clear correlation between calli induction and plant regeneration response was observed (Fig. 3), these two wild forms regenerated plants from anther culture with the highest efficiency probably suggesting that either this capacity was lost or the genes responsible for an increased in vitro regeneration competence were switched off during the domestication of these species. The genetic determinism of regeneration competence is still poorly understood; however, a number of genes have been identified that positively influence the competence of plant cells for somatic embryogenesis and/or adventitious shoot formation (Horstman et al. 2017; Kumar and Van Staden 2019; Meng et al. 2019). Most of these genes have been identified in *Arabidopsis* and many of them encode transcription factors or proteins involved in signal transduction (Jha et al. 2020). Seguí-Simarro (2010) proposed that androgenesis perhaps is a developmental pathway based on this capacity of ancient plant relatives, and currently displaced by the evolutionary advantages of sexual reproduction. Thus, when the normal process of sexual reproduction is blocked or disturbed by isolating anthers, the plant will activate this ancient mechanism in order to ensure reproduction by any means.

In line with our results, peanut androgenesis was induced in the anther cultures of the cultivated species (*Arachis hypogaea*) and a diploid wild species (*A. villosa*). Even though the anthers of *A. villosa* underwent little proliferation to form calli, they regenerated a high percentage of shoots and plantlets (40–70%), whereas in *A. hypogaea*, there was a profuse proliferation to form a mass of callus but shoots were only occasionally formed (< 18%) (Bajaj et al. 1981). In solanaceous crops like potato (*Solanum tuberosum*), the induction of androgenesis by anther cultures of potato relatives such as *S. phureja* (Teparkum and Veilleux 1998), *S. acaule* (Rokka et al. 1998) and *S. chacoense* (Hermsen 1969) has been reported. Likewise, in tomato, Reynolds (1990) reported the production of callus, embryoids and regenerated plants from cultured anthers of wild tomato (*S. carolinense*) while all evaluations of the androgenic competence in commercial tomato cultivars to date showed null or very few positive results (Seguí-Simarro 2016). Conversely, with eggplant, androgenetic responses were only found in the cultivated form suggesting that unsought selection may have been done for higher androgenic response in breeding programs (Salas et al. 2011).

In pea the evolution of androgenesis has not been studied yet, even if evolutionary studies for other traits could help to corroborate our assumption. For example, evolution of early flowering under domestication was studied by Weller et al. (2012), who performed genetic analysis of the differences in flowering and photoperiod responsiveness between wild and domesticated pea and indicated a loss of function in genes

controlling responsiveness to photoperiod or vernalization. These changes reduce the length of the growth cycle, permitting a shift from winter to spring cropping, the latter being found in cultivated pea types. Other traits selected during domestication and development of modern cultivated types include those that are determined by one or a few genes, such as "a" (lack of anthocyanin production) and "r" (wrinkled seed in garden types), which improved palatability, and "p" and "v" for the absence of sclerenchymatic tissue in pods (Smýkal et al. 2018b). Both morphological and genetic studies have identified *P. sativum* subsp. *elatius* and *P. fulvum* as wild germplasm in that they have dehiscent pods and seed dormancy (thick testa). In contrast, *P. sativum* subsp. *sativum* (including varieties *arvense*, *transcaucasicum* and *asiaticum*) are diagnosed by characters that are selected during domestication, namely: non-dehiscing pods, absence of seed dormancy and seeds with a smooth, thin testa (Trněný et al. 2018). Domestication has also resulted in increased seed and pod size in pea (although not as markedly as in other crops) with a correlated increase in leaf size and stem strength (Weeden 2018). Also many of protease inhibitors have been reduced or eliminated during the domestication process (Smýkal et al. 2018b).

*Pisum sativum* is now generally viewed as a complex species that includes a wide variety of cultivated forms beside the wild form (Smýkal et al. 2017; Trněný et al. 2018). With regard to the different genotypes cultivated of *P. sativum* in our study, the botanical variety *arvense* regenerated plants with the highest percentage (40%) being a good candidate to study androgenesis, at least in the conditions we used.

When comparing the different commercial varieties, some of them failed to regenerate shoots from the calli and others regenerated but with low frequencies demonstrating that the different pea genotypes are recalcitrant to anther culture. These results agree with those reported by Ochatt et al. (2009) who indicated that whatever the basal medium, treatment or culture conditions, genotype is the main factor governing androgenetic capacity in pea, when microspores are not exposed to any specific treatment. The commercial varieties showing the best performance in our studies were Primogénita FCA-INTA and B101 in terms of both calli and plant production. Also, they showed the best grain yield, earliness and morphological characteristics in the field as indicated INTA (2019).

In breeding, it is usual to exploit the natural variability present in related species. Wild relatives offer tremendous opportunities for improving a number of traits in cultivated legumes. Many interesting traits such as biotic and abiotic stress tolerance/resistance have been identified in wild relatives and introgressed in cultivated pea (Pratap et al. 2018; Coyne et al. 2020). Based on results obtained here, we considered that the wild species *P. fulvum* and the wild form *P. sativum* subsp. *elatius* were highly responsive genotypes to anther culture, whereby it would be important to use these wild form and species as donor parents in hybridizations with *P. abysinicum* or with different commercial varieties of *P. sativum* to improve their androgenetic competence. In other species, such as with eggplant, Chakravarthi et al. (2010) established that some traits related to in vitro culture (callus initiation, embryogenic callus percentage, mean number of regenerated shoots per callus) are easily transferable from related species to economically important, recalcitrant cultivars. They showed that additive gene action was predominant for the in vitro characters and the regeneration of shoots from explants appears to be under strong genetic control. In another anther culture study, barley and potato high-responding genotypes were crossed with commercial varieties. The $F_1$ generation showed an intermediate reaction between both parents, suggesting that the high response to anther culture is heritable (Wenzel and Foroughi-Wehr 1984).

Within cultivated forms of *P. sativum*, it would be useful to explore the possibility of making crosses with *P. sativum* var *arvense* to transfer its androgenic competence to recalcitrant genotypes of agronomic interest such as genotypes Primogénita FCA-INTA and B101. In doing so, it could be possible to obtain then pure lines that have earliness, and high yield from the genotypes Primogénita FCA-INTA and B101. Başay and Ellialtioğlu (2013) determined the androgenic capacity of some eggplant (*Solanum melongena* L.) commercial varieties and breeding lines, and established that androgenesis may be induced in the less responsive genotypes by crossing them with more responsive genotypes.

Although the plants were regenerated with an uncertain knowledge of their origin (i.e. haploid microspore vs. somatic anther wall tissue), the ultimate goal of our work was to identify within the genus *Pisum* potential sources of androgenic competence to introduce it into recalcitrant commercial varieties (which, to our knowledge, has not been achieved to date).

The present results are consistent with previous reports of difficulties in obtaining confirmed haploid shoots or plantlets, with most results stopping short of the recovery of pea plants (Croser et al. 2005, 2007; Sidhu and Davies 2005), irrespective of the use of male organs (anthers) or reduced gametophytes (microspores) as starting material. Based in the pea bibliography to date (Gupta 1975; Croser and Lülsdorf 2004; Sidhu and Davies 2005; Ochatt et al. 2009; Bobkov 2010, 2014; Lulsdorf et al. 2011; Ribalta et al. 2012) where the recovery frequency of confirmed haploid pea plants through anther or microspore culture has been about 4% and 20% we could estimate the frequency of putative haploid plants that we would have obtained. The number of haploid plants ranged from 2 to 10 highlighting the genotypes of *P. fulvum*, *P. sativum* subsp. *elatius* and *P sativum* subsp. *sativum* var *arvense*.

Likewise, the confirmation of their haploid origin using an optimized methodology such as flow cytometry (Ochatt 2008; Ochatt et al. 2011; Ribalta et al. 2012) is an important challenge that must be addressed before pea androgenesis can be exploited in genetic breeding.

Data presented here form a solid basis for further efforts (currently under way) designed to improve these responses and also to extend the strategies developed to other genotypes and to microspores of these species. This investigation is part of an effort we are carrying to obtain more information on the androgenic competence of genus *Pisum*. It is our hope that our results will shed some light on this slow-developing but important field of research.

## Conclusions

There was a great variation in the androgenic response among different genotypes in the genus *Pisum* exposed to the same set of inductive and cultural conditions.

The wild species *P. fulvum* and the wild form of *P. sativum (P. sativum* subsp. *elatius)* were identified as potential sources of androgenetic competence useful to exploit and transfer this trait to the cultivated forms.

*Pisum sativum* subsp. *sativum* var *arvense* could be used to optimize the conditions for the anther culture protocol prior to widening its application to other genotypes, and also to induce androgenesis by crossing with recalcitrant commercial varieties.

**Acknowledgements** This research was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), Instituto de Investigaciones en Ciencias Agrarias de Rosario (IICAR-CONICET) and Fondo para la Investigación Científica y Tecnológica (FONCyT, Argentina).

**Funding** This study was funded by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), Instituto de Investigaciones en Ciencias Agrarias de Rosario (IICAR-CONICET) and Fondo para la Investigación Científica y Tecnológica (FONCyT, Argentina).

**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

**Code availability** Not applicable for that section.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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