Journal of Economic Entomology, 111(3), 2018, 1023–1029
doi: 10.1093/jee/toy081
Advance Access Publication Date: 31 March 2018
Research Article

Apiculture and Social Insects

Onion Hybrid Seed Production: Relation with Nectar Composition and Flower Traits

Veronica C. Soto,[1] Cristian A. Caselles,[2] Maria F. Silva,[1] and Claudio R. Galmarini[3,4,5,6]

[1]IBAM, UNCuyo, CONICET, Facultad de Ciencias Agrarias, Almirante Brown 500, M5528AHB, Chacras de Coria, Mendoza, Argentina; [2]Monsanto Argentina SRL, Ángel Mosconi 627, Luján de Cuyo, Mendoza, Argentina; [3]Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria La Consulta, CC8 (5567), La Consulta, Mendoza, Argentina; [4]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina; [5]Facultad de Ciencia Agrarias, Universidad Nacional de Cuyo, Almirante Brown 500, M5528 AHB, Chacras de Coria, Luján, Mendoza, Argentina.; and [6]Corresponding author, e-mail: galmarini.claudio@inta.gob.ar

Subject Editor: John Trumble

Received 19 January 2018; Editorial decision 7 March 2018

Abstract

Onion (*Allium cepa* L.) is one of the main vegetable crops. Pollinators are required for onion seed production, being honeybees the most used. Around the world, two types of onion varieties are grown: open pollinated (OP) and hybrids. Hybrids offer numerous advantages to growers, but usually have lower seed yields than OP cultivars, which in many cases compromise the success of new hybrids. As pollination is critical for seed set, understanding the role of floral rewards and attractants to pollinator species is the key to improve crop seed yield. In this study, the correlation of nectar-analyzed compounds, floral traits, and seed yield under open field conditions in two experimental sites was determined. Nectar composition was described through the analysis of sugars, phenol, and alkaloid compounds. Length and width of the style and tepals of the flowers were measured to describe floral traits. Floral and nectar traits showed differences among the studied lines. For nectar traits, we found a significant influence of the environment where plants were cultivated. Nonetheless, flower traits were not influenced by the experimental sites.The OP and the male-sterile lines (MSLs) showed differences in nectar chemical composition and floral traits. In addition, there were differences between and within MSLs, some of which were correlated with seed yield, bringing the opportunity to select the most productive MSL, using simple determinations of morphological characters like the length of the style or tepals size.

Key words: *Allium cepa* L., seed production, crop pollination, male sterility

Onion (*Allium cepa* L.) is one of the most important species of the *Allium* subgenus belonging to Alliaceae family. Each onion umbel has hundreds of perfect flowers. Anthers release pollen before the stigma is receptive. Protandry encourages the out-crossing among onion plants (Currah and Ockendon 1978, Cebeci and Hancı 2016).

Around the world, two types of onion varieties are commonly grown: open pollinated (OP) and hybrids (Sidhu et al. 2004). Hybrids offer more vigor, uniformity, and combine better biotic and abiotic stress resistance than OP materials, and provide intellectual property to breeders (Cebeci and Hancı 2016, Colombo and Galmarini 2017). For hybrid seed production, CMS-based systems are used worldwide (Havey 2000). Two main sources of CMS have been genetically characterized, identified as S and T (Havey 2000). The S type is the most commonly used owing to the usual occurrence of the recessive allele at *Ms*, and stability over environments (Havey 2000).

For hybrid seed production, it is necessary to cross a male-sterile line (MSL) with a fertile one. Commercial onion hybrid breeding requires the development of selected MSLs, related inbred maintainers and inbred pollinator lines, with good specific combining ability.

Nearly two-thirds of onion cultivars in catalogs of the most important seed companies belong to the hybrid category (Cebeci and Hancı 2016). Nevertheless, seed companies reports and previous studies have mentioned that hybrid seed yield is erratic and considerably lower compared with the OP varieties (Soto et al. 2013). Moreover, it has also been observed a highly variable seed yield among different onion hybrids. Low onion seed yield can compromise the success of new hybrids. Poor seed yields often are due to poor pollination (Hagler 1990; Hagler et al. 1990; Silva and Dean 2000; Silva et al. 2003, 2004; Marino et al. 2013). Honeybees (*Apis mellifera* L.) are the most efficient and practical pollinator of this crop because they have a great affinity for pollen and nectar and transfer pollen efficiently (Williams and Free 1974). Therefore, increasing seed yield depends heavily on increasing activity by these cross-pollinating insects (Abrol 2010b).

Plants offer nectar as reward (Nicolson 2011). Sugars are the main compounds in nectar and they are the major energy source for pollinators (Amato and Petit 2017). Besides, nectar contains a variety of minor solute compounds, such as amino acids, lipids, proteins, phenols, and alkaloids (Torres and Galetto 1998). It has been reported that nectar secondary compounds may provide reproductive benefits to plants; nevertheless, it is expected to have detrimental effects if pollinators are deterred from visiting flowers (Gegear et al. 2007, Steverson et al. 2017). Thus, chemical data describing nectar composition beyond the primary sugar components are necessary to understand the pollinator reaction to the food source. To the present, the relationship between nectar composition and pollinator attraction has been poorly studied in onion. However, the lack of attractiveness has been historically attributed to high nectar potassium concentration, sugar composition, and nectar volume (i.e., onion MSL flowers produce less nectar) (Hagler 1990, Silva and Dean 2000, Nicolson and Thornburg 2007, Abrol 2010a). In a previous study, Soto et al. (2013) in concordance with the results published by Silva and Dean (2000) found that although potassium is always present in high concentrations in onion nectar, this compound was not correlated with poor seed yield of hybrid lines.

Flower traits such as size, color, nectar volume, and composition are considered as the most important factors to attract honeybee (Muth et al. 2016). In onions, the size of anthers and tepals seem to be the most significant traits for bee attraction (Soto et al. 2013). Lines with bigger tepals and shorter styles had higher seed yields (Soto et al. 2013). In previous studies carried out inside cages, nectar profile showed qualitative and quantitative differences, as well as in flower traits among MSLs and between OP and MSL. However, conditions inside pollination cages differ from ambient field conditions, which could affect both onion physiological and morphological development and pollinator behavior. Also, the temperature inside cages is higher than that in open field conditions, and can affect bee behavior (Soto et al. 2013). Therefore, this study was conducted to determine the correlation of nectar-analyzed compounds, floral traits (i.e. tepal and style size), and seed yield under open field conditions. Two locations in the main seed production area of Argentina were chosen for that purpose. This work may contribute to select MSLs that could produce higher seed yields.

Materials and Methods

Plant Materials

Eight MSLs obtained from Monsanto SRL (Mendoza, Argentina), and an OP onion cultivar, Valcatorce INTA (Galmarini 2000) were used in the experiments (Table 1). In early March 2015, 150 bulbs per replicate from the different MSLs were planted in 2 locations using 3 replicates in a randomized complete block design. These bulbs were distributed in three rows of 5 m long, planting 10 bulbs per meter. The furrows were about 1 m apart. Bulbs of the fertile line were planted on the side of each block in a single line. The ratio between sterile and fertile plants was 3:1, which is the normally used ratio in a commercial crop. Each material, including all the MSLs and the OP cultivar were coded 1 through 9 (Table 1). Pesticides were not used along the experiment, and plants were watered by drip-irrigation system.

The plants flowered from October to November 2015. Flowers were collected at 50% bloom of the umbel. To perform this, at midday, 5 flowers from 10 umbels per plot were randomly chosen.

Experimental Sites

Onion lines were grown in two different places, EEA INTA Mendoza (33°0′21.57″S, 68°51′40.86″W), Mendoza (A) and Villa Tacu, Zonda, San Juan, Argentina (31°32′10.38″S 68°42′21.97″W) (B), during 2015. The second location is ~160 km to the north from the first one and blooming there occurs between 20 and 30 d earlier than in the site A.

Nectar Extraction

Since onion flowers are small and produce a small amount of nectar, the collection of nectar was achieved by removing anthers, filaments, and peduncle from freshly opened flowers belonging to umbels that were between 40 and 60% of flowering. Following excision from umbel, flowers were centrifuged in a 1.5 ml microtube at 13,000 rpm, and 4°C during 30 min, to extract nectar. An average of 100 µl of nectar was extracted from all the opened flowers of 10 umbels per plot. Nectar was preserved at −80°C until analysis.

Bee Foraging Behavior

Apis mellifera L. was used for crop pollination. Standard hives, i.e. 10 frame Langstroth beehives, were placed in the field at 10% of flowering. Plots of 1 m were marked randomly in every block along rows. A visual counting method was used to record the number of bees visiting each plot for 1 min. The mean of three observations from each side of the plot constituted a reading for each line. Every day at midday, up to 100% bloom the number of bee visits per umbel and minute was recorded.

Environmental Factors

Sensors were placed to record air temperature (°C) and relative humidity (%) every 30 min (HOBO U23 Pro V2 External Temperature/Humidity Data Logger; Onset, Bourne, MA, USA). Cloud cover (in oktas) and wind speed (Beaufort scale) were recorded.

Seed Yield

When seed development was completed, umbels were harvested and dried for 21 days. The umbels were spread in a 20-cm thick layer and sundried. Umbels were turned regularly to achieve a uniform drying and to avoid rotting. Humidity was controlled every 2 d with a humidity sampler John Deer calibrated for onion and when the average humidity reached 14%, seed threshed was performed by hand. Seeds were cleaned by hand, collected in bags, and weighed to calculate seed yield.

Nectar Chemical Analysis

Sugar analysis was done on a Shimadzu HPLC, model 10 A, with a refractometric index detector and a Rheodyne injector with 20-µl sample loop. An alkylamine column (5 µm, 250 × 4.6 mm) was used with a mobile phase of water: acetonitile (80:20 V:V) at 1 ml/min. Concentration of sugars was expressed as grams per litre of nectar (OIV 2003).

Alkaloid and phenol compounds were extracted according to Soto et al. (2014, 2016). Phenolic and alkaloid compounds were determined using an HPLC system (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany) with a Dionex MWD-3000 (RS) detector and a Chromeleon 7.1 software. The working wavelengths for the different analytes were 254, 280, 320, and 370 nm. HPLC separations were carried out in Zorbax SB-Aq column (4.6 × 150 mm, 5 µm) Agilent Technologies, California, USA. Ultrapure water with 0.1% formic acid (A) and acetonitrile (B) was used as mobile phases. Alkaloids and phenols were separated using the following gradient: 0–2.7 min, 5% B; 2.7–11 min, 30% B; 11–15 min, 95% B: 15–20 min 5% B at 1 ml/min. The column temperature was held at 20°C and the injection volume was 5 µl. Catechin, rutin, naringenin, cinnamic acid, syringic acid, quercetin, luteolin, chlorogenic acid, apigenin, vanillic acid, caffeic acid, nicotine, theobromine, theophylline, caffeine, harmaline, and piperine were determined (Sigma-Aldrich, St. Louis, MO). All the nectar analyses were done in triplicate. Concentrations of phenolic and alkaloid compounds were expressed as milligrams per litre of nectar.

Floral Traits

Five plants per block of each line were selected randomly, taking 10 flowers per plant. Flowers were kept in formaldehyde–acetic acid–alcohol solution, in a proportion of 10, 5, and 50%, respectively, and 35% of water, until morphology measurements. For each flower, style length, as well as length and width of tepals were measured with a digital camera (Micrometrics 318 CU, Shanghai, China) mounted on a stereomicroscope (Nikon Eclipse E200, Tokyo, Japan).

Statistical Analysis

Values were expressed as means ± SEM. Data were analyzed by analysis of variance (ANOVA) to test the significant differences. Means were compared using Tukey's test. The results were considered significant at $P \leq 0.05$ unless specified otherwise. Principal component analysis was applied to highlight the data structure and to find the overall relationships between nectar composition and floral traits that condition the pollination efficiency of MSLs for onion hybrid seed production. Basic statistic and multivariate analysis were performed using statistical package InfoStat2016 for Windows (Cordoba, Argentina).

Results

Seed Yield

No significant interaction was found between seed yield and locations. Large differences in seed yields were found between the hybrids produced using different MSLs and the fertile line. Moreover, there were differences among the hybrids produced using different MSLs. MSL 8 and 9 were associated with the lowest yields. These lines had up to 15% less seed yield than line 1, which was the MSL with the highest seed yield (Table 1).

Bee Activity and Environmental Factors

Among climate factors, relative humidity and temperature had more influence on bee activity than rain or wind during blooming (Fig. 1). At both sites, we observed that humidity was negatively correlated with bee activity. When the levels of relative humidity were high, bees did not fly and visits were null.

On another hand, bloom percentage had a great influence on bee activity (Fig. 1) and was higher when it was between 50 and 70% (data not shown). For bees, in general, female lines were less attractive than the male lines (Fig. 2). Visits observed at 50% of flowering in all studied lines showed a relationship with yields ($r = 0.69$) in both locations.

Nectar Composition

Nectar contained predominantly hexoses with lower levels of sucrose. Fructose was the sugar that was in higher concentration. The percentage ratio of fructose, glucose, and sucrose was relatively constant among MSLs (50% fructose, 30% glucose, and less than 10% sucrose) (Fig. 3).

Phenol and alkaloid compounds showed quantitative and qualitative differences among lines and experimental sites ($P \leq 0.05$). All the studied compounds concentrations were higher in San Juan (location B) (Fig. 3). The OP line showed a higher concentration of methylxanthines.

In all cases, the ANOVA revealed significant differences for nectar compounds among lines and locations. The line × location interaction was significant at $P < 0.05$ showing that the location where umbels grow have a major role in nectar composition, affecting

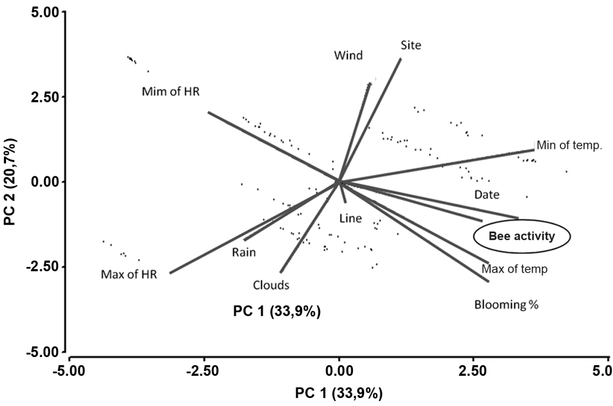

Fig. 1. Principal component analysis plot of nine onion lines calculated on the environmental factors, rainfall (PP) (mm), maximum (Max of Temp) and minimum (Min of Temp) temperatures (°C), maximum (Max of HR) and minimum (Min of HR) relative humidity (%), wind speed (Beaufort scale), Cloud cover (oktas), and bee activity (number of visits per umbel) during crop season for locations A and B.

Table 1. Characteristics and seed yield (average of both locations) of the studied onion lines

Line	Bulb color	Day length	Bolting	Bulb shape	Seed Yield (g/umbel)
OP4	White	Short day	Late	Globe	5.24 ± 0.21 a
MSL3	Yellow	Short day	Late	Flat	3.98 ± 0.30 b
MSL6	Yellow	Intermediate day	Early	Globe	3.97 ± 0.32 b
MSL1	Red	Short day	Early	Globe	3.94 ± 0.11 bc
MSL7	Yellow	Short day	Medium	Globe	3.94 ± 0.27 bc
MSL2	Yellow	Short day	Medium	Flat	3.86 ± 0.16 bc
MSL5	White	Short day	Late	Globe	3.74 ± 0.24 bcd
MSL8	White	Short day	Late	Globe	3.53 ± 0.17 cd
MSL9	Yellow	Short day	Late	Globe	3.33 ± 0.36 d

Values represent mean ± SD of three determinations. Values in the same column with different letters present significant differences $P \leq 0.05$.

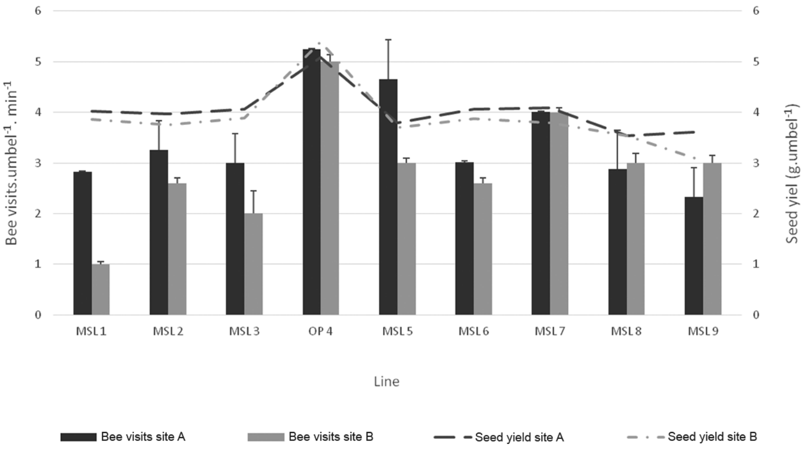

Fig. 2. Number of bee visits at 50% of blooming of different onion lines and seed yield in both studied locations (A in dark gray and B in light gray).

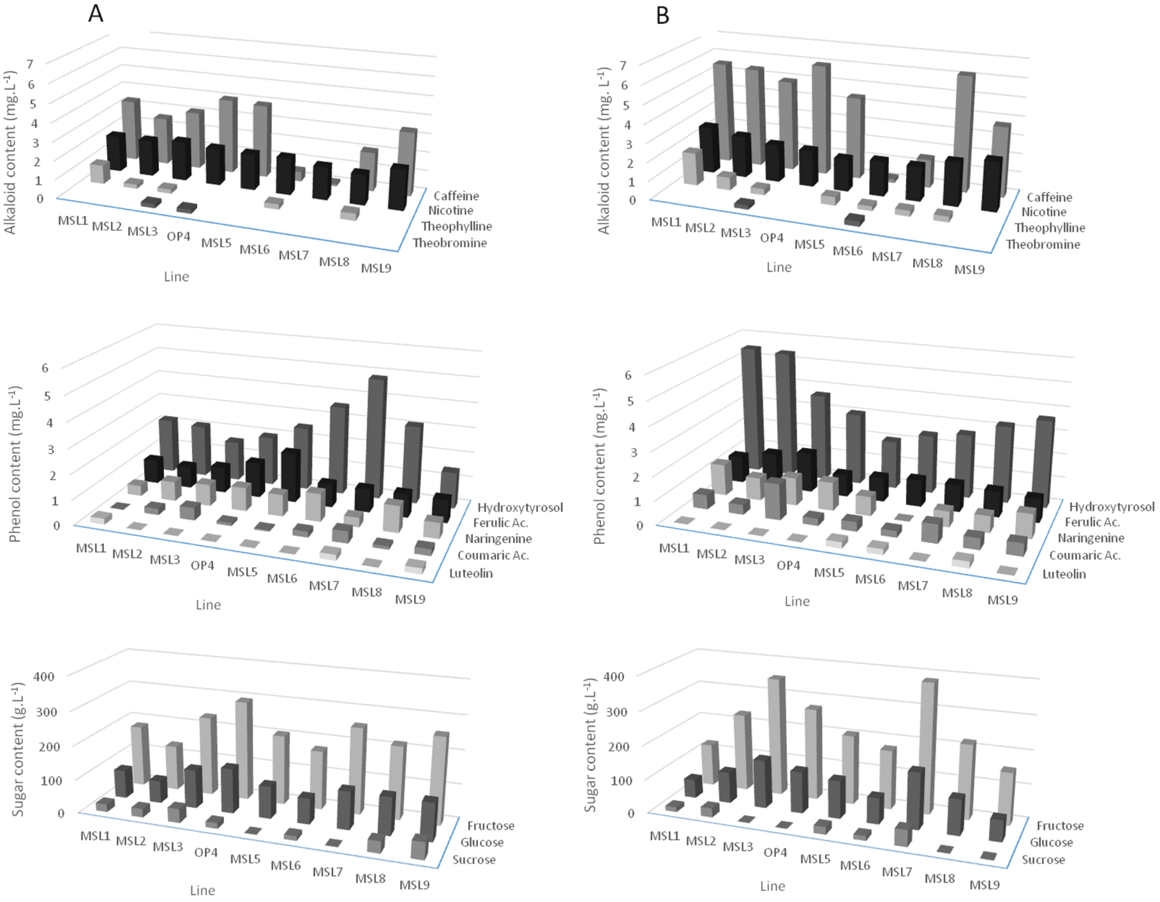

Fig. 3. Nectar alkaloid, phenol, and sugar concentration of different onion lines for the two experimental sites. Phenolic and alkaloid content is expressed as milligrams per litre of nectar. Sugar content is expressed as grams per liter of nectar.

the profile and quantity of the compounds present in nectar. Data obtained showed that nectar profile and concentration strongly depended on meteorological conditions. All studied compounds showed an interaction with the experimental sites. Depending on the site of study, correlations between nectar compounds and seed yield differ (Table 2). Despite sugars having a strong influence on bee visits, in both locations we found that other compounds and particularly flower traits had positive and negative correlations with the number of bee visits and seed yield, while other were not significant (Fig. 4). Glucose and fructose were the nectar compounds that were strongly correlated with bee visits. Nevertheless, floral traits had the strongest influence in bee visits and seed yield.

Floral Traits

We found that flower traits do not interact with the experimental sites. The different studied lines represented the major source of variability. Internal and external tepals, as well as the style length, showed significant differences between the OP and MSLs, and among MSLs (Table 3). The OP line has the shortest style among the studied material (4.99 ± 0.08 mm), while line 8 had the longest style (5.79 ± 0.26 mm). However, the OP line showed the largest internal tepal width (3.15 ± 0.12 mm) and the MSL 9 had the shortest (2.60 ± 0.1 mm). The onion lines with a longer style had fewer bee visits and less seed yields (Fig. 4). Conversely, tepal length and width have a significant positive relationship with seed yield (Table 2).

Table 2. Correlation between environmental factors, nectar, floral traits, and seed yield

	Experimental site	
	A	B
Trait	Seed yield	
Maximum relative humidity	0.45^{*}	-0.28^{ns}
Minimum relative humidity	-0.73^{***}	0.13^{ns}
Maximum temperature	-0.03^{ns}	0.12^{ns}
Minimum temperature	-0.43^{*}	0.44*
Rain	0.00^{ns}	0.01^{ns}
Clouds	-0.63^{***}	0.00^{ns}
Fructose	0.35^{ns}	0.29^{ns}
Glucose	0.42^{*}	0.28^{ns}
Sucrose	-0.31^{ns}	-0.12^{ns}
Theobromine	0.65^{***}	-0.07^{ns}
Theophylline	-0.14^{ns}	-0.39^{*}
Caffeine	0.18^{ns}	-0.03^{ns}
Nicotine	0.13^{ns}	-0.18^{ns}
Hydroxytirol	-0.07^{ns}	-0.11^{ns}
Naringenine	0.04^{ns}	0.21^{ns}
Coumaric acid	-0.01^{ns}	-0.20^{ns}
Ferulic acid	0.19^{ns}	-0.15^{ns}
Luteolin	-0.18^{ns}	-0.19^{ns}
Style width	*-0.10^{ns}*	
Style length	*-0.45^{***}*	
External tepal width	*0.44^{***}*	
External tepal length	*0.14^{ns}*	
Internal tepal width	*0.70^{***}*	
Internal tepal length	*0.45^{***}*	
R style length/External tepal width	*-0.60^{***}*	
R style length/External tepal length	*-0.52^{***}*	

R, ratio.

$^{\mathrm{ns}}$,*, **, ***Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. For floral traits (highlighted) there was not interaction with the experimental site, so average for both locations is indicated.

Discussion

The environment is expected to have a great influence on onion performance and it will be initially reflected in variation at the individual level (Brown et al. 1977, Canto et al. 2011). We found that environmental factors had influence on bee visits as it was expected, since they affect directly bee foraging behavior and indirectly exert influence on plant physiology. Factors such as temperature, light, CO_2 levels, and water availability may affect nectar composition in a direct or indirect way (Chalcoff et al. 2017). Relative humidity had the greatest influence on bee foraging behavior. These results agree with those reported by Abrol (2010b), who found that bee visits were positively influenced by temperature, light intensity, and sugar concentration variability, but were related in the opposite way to relative humidity. Moreover, these results agree with those reported by Silva et al. (2004), who observed that relative humidity was correlated with nectar production on both hourly and daily bases. However, onion nectar production showed an opposite relationship to relative humidity, at higher relative humidity, nectar production declines, which could directly affect bee behavior. The OP line was the most visited by bees while there were some MSL that showed significant differences with the fertile line. These data obtained under open field conditions are consistent with those previously found by our group under cage conditions (Soto et al. 2013). Although, bee visits and seed yield were highly correlated ($r = 0.69$) in both sites, seed yields were not influenced by the location. This fact suggests that onion seed yield depends more on the genotype than the environmental conditions.

The environment seems to have a great influence in nectar composition. Nectar production is a multigene trait that can be affected by selective breeding programs (Silva et al. 2004). Temperature, solar radiation, vapor pressure, and soil moisture may affect nectar quantity and quality and the volatile compounds produced by the flower. These effects may be further heightened depending on the genotype by environment interactions (Silva et al. 2004). All nectar compounds varied with the line and the studied site. Nectar major compounds were hexoses and among these, fructose represented around 50% while sucrose was much lower. These results agree with Silva and Dean (2000) and Hagler et al. (1990), who showed

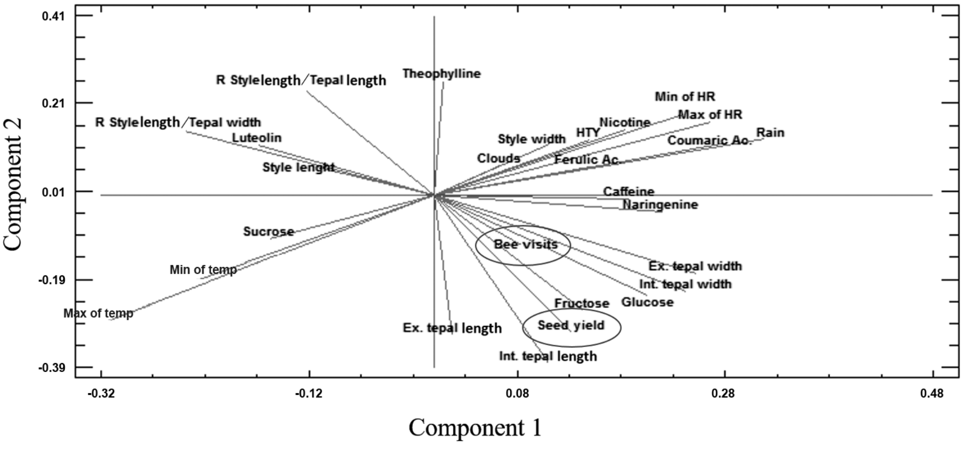

Fig. 4. Principal component analysis plot of nine onion lines calculated on the basis of their chemical and morphological information from nectar and flowers and the effect of environmental factors. HR, relative humidity; HTY, hydroxytyrosol.

Table 3. Floral traits of different onion lines expressed as millimeters

Line	Style W	Style L	External tepal W	External tepal L	Internal tepal W	Internal tepal L
MSL1	0.54 ± 0.03 ab	4.50 ± 0.32 e	2.54 ± 0.09 c	4.75 ± 0.18 d	2.70 ± 0.08 b	5.02 ± 0.16 d
MSL2	0.50 ± 0.02 cde	5.60 ± 0.12 ab	2.68 ± 0.03 b	5.02 ± 0.04 c	2.70 ± 0.08 b	5.31 ± 0.10 cd
MSL3	0.53 ± 0.02 bc	5.18 ± 0.15 cd	2.62 ± 0.05 bc	5.13 ± 0.12 bc	2.69 ± 0.07 b	5.41 ± 0.09 bc
OP4	0.48 ± 0.01 e	4.99 ± 0.08 d	2.79 ± 0.04 a	5.34 ± 0.09 ab	3.15 ± 0.125 a	5.90 ± 0.19 a
MSL5	0.48 ± 0.01 de	5.47 ± 0.20 abc	2.33 ± 0.04 e	4.98 ± 0.10 cd	2.54 ± 0.08 b	5.16 ± 0.08 cd
MSL6	0.51 ± 0.09 bcd	5.31 ± 0.18 bcd	2.40 ± 0.04 e	5.05 ± 0.11 c	2.63 ± 0.06 b	5.07 ± 0.13 d
MSL7	0.56 ± 0.08 a	5.05 ± 0.08 d	2.43 ± 0.02 de	5.39 ± 0.15 a	2.57 ± 0.07 b	5.77 ± 0.22 a
MSL8	0.48 ± 0.09 de	5.79 ± 0.26 a	2.58 ± 0.08 bc	5.42 ± 0.20 a	2.62 ± 0.12 b	5.63 ± 0.21 ab
MSL9	0.50 ± 0.01 cde	5.50 ± 0.13 abc	2.52 ± 0.05 cd	5.17 ± 0.11 abc	2.60 ± 0.10 b	5.20 ± 0.12 cd

L, length; W, width.

Values represent mean of the two locations studied ± SD of three determinations. Values in the same column with different letters present significant differences $P \leq 0.05$.

that the concentration of sucrose in onion nectar was always 3- to 6-fold lower than fructose. As well as sugars, phenol and alkaloid concentrations showed differences between locations. Both, phenol and alkaloid compounds have been stated as deterrents or repellents of pollinators (Gegear et al. 2007, Nicolson and Thornburg 2007, Adler and Irwin 2012, Köhler et al. 2012, Mustard et al. 2012, Steverson et al. 2017). Nonetheless, the OP line, which is the line with more pollinator visits and higher seed yield, showed a higher concentration of methylxanthines, and in the experimental site A, this line showed theobromine. This could suggest that the attractiveness of onions is mainly determined by the amount of beneficial compounds such as carbohydrates present in the nectar for the pollinators (Soto et al. 2016). In spite of some coincidences with our previous studies (Soto et al. 2013, Soto et al. 2014, Soto et al. 2016), there were some differences because these studies have been carried out in one location and under cages.

Bee visitation frequency and plant fitness are determined by the integration of multiple factors, such as the amount of a reward, pollen and nectar, and/or fragrance (Fernández et al. 2009, Muth et al. 2016). Volatile organic compounds (VOCs) play an important role on bee attraction as well as defending plants against herbivores and pathogens (Li et al. 2006). In onion, VOCs found in great quantity, such as the organosulfur compounds, have been reported as repellents (Soto et al. 2015). The amount of these compounds in the different lines may influence on bee visits.

One of the roles of floral phenotype is to signal that there are nutritious or reproductive rewards to pollinators (Raguso 2004). Other factors that could affect pollination as well are flower color and the size of each floral trait. Their variability among different flowers can be related to the reproductive success (Fernández et al. 2009). According to our results, the size of the tepals is possibly playing an important role in this signaling process. It is notable that the onion lines with a longer style had fewer bee visits and less seed yields. This fact could be related to heterostyly, which plays a role in pollinator attraction in many species (Barrett 1992). This is a genetic polymorphism where the heights at which the stamens and style are positioned differ between individual plants of a species but has not been described in onion flowers yet. Style length and tepal length showed a negative and positive significant high correlation, respectively, with seed yield. All the carried out experiments in this study showed that the MSLs have differences in floral traits from OP onion cultivar and those lines with bigger tepals attract more bees, thus have a higher seed yield. These results are in agreement with those previously reported by our group (Soto et al. 2013), where we found that these are the traits that show more variation between OP and MSL, and among MSL. It is noteworthy to mention that data obtained with onions cultivated under a cage, under controlled conditions, are consistent with data obtained in this study, where more lines were studied in two different experimental sites under an open field conditions and we could confirm that these characters are not influenced by the location where onions are cultivated.

The correlation found in some floral and chemical traits with onion seed yield may be a great contribution for onion breeders, who could select lines that have promising traits for pollinator attraction, and as consequence, better seed yields. Moreover, this study contributes to understand the factors that affect bee foraging for onion hybrid seed production. Further studies are required concerning floral traits with regard to their heritability, to evaluate if the characteristics favored in the generation of the parents are expressed in the resulting offspring.

Acknowledgments

We thank Monsanto SRL for providing seed and plant materials and Kelli Lynn Sansossio for English revision. This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (PICT-2015-0358), CONICET (11220150100817CO), INTA PNHFA 1106071.

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