

Physiological and Technological Consequences of Benzyl Adenine (BAP) Application on Butternut Squash (*Cucurbita moschata* Duchesne ex Poir.) Productivity

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Some authors have indicated that the reason why benzyl adenine (BAP) stimulates dry matter accumulation in squash and pumpkin is unclear, but we believe that two possibilities are likely: (i) higher photo-assimilate partitioning towards the shoot; or (ii) increased in the photosynthetic efficiency of leaves. Thus, the aim of this work was to study the effect of an exogenous BAP spray on biomass accumulation and yield in pumpkin plants grown in commercial facilities. Our results showed that, the relative growth rate of Butternut squash plants grown under commercial plant

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density did not change and that yield was the same in both BAP-sprayed and control plants. However, a single BAP application decreased leaf size and total leaf area per plant but increased the rate of leaf appearance. Our findings suggest the possibility of an increase in plant populations through changes in crop architecture and an increase in yield on a field area basis.

Keywords: Cytokinin; crop architecture; fruits; growth; vegetables.

1. INTRODUCTION

The increasing consumption of fresh fruits and vegetables due to the growing population is currently showing unprecedented demands [1]. Future demand for vegetables will increase over the coming decades, driven by population growth and changing dietary habits, which means that vegetable production will have to increase as well [2].

Butternut squash (*Cucurbita moschata* Duchesne ex Poir.) and other cucurbits are major vegetable crops grown in almost all arable regions of the world. They are grown both for human consumption of their fruit, which is a rich source of valuable nutrients such as vitamin C, thiamine, riboflavin, niacin, vitamin B6, folates, vitamin E, vitamin K, β -carotene, potassium, phosphorus, magnesium, iron, and selenium [3,4,5] and for their medicinal properties [6]. However, nutrient compositions differ considerably among squash-pumpkin species and parts [7].

The most important factors determining pumpkin crop productivity are the amount of photosynthetically active radiation (PAR) intercepted by the plant canopy during the growing season, the efficiency with which the light energy is converted into plant biomass, and the proportion of biological yield that is allocated to economic yield. However, only few works have described yield components and improved productivity in this species [8,9].

Because the high squash-pumpkin relative growth rate (RGR) and a crop architecture that allows achieving the critical leaf area index (LAI) quickly, the current densities suggested range between 3,000 and 9,000 plants ha^{-1} (0.3 to 1.2 m in-row plant spacing) depending on the variety, equipment and available land [10]. These densities have not been changed during the last 20 years [11,12] because increased plant population results in a significantly linear decrease in average fruit size [10].

Hassan and Chaudhry [13] studied the effect of growth hormones such as kinetin on the external

and internal morphology of *Cucumis sativus* and found that this growth regulator increases both fresh and dry weight but decreases the length of internodes and petioles. On the other hand, Partier et al. [14] treated excised *Cucurbita* cotyledons with other exogenous cytokinin such as benzyladenine (BAP) and found a marked stimulation of Rubisco activity, enzyme protein content, and incorporation of labelled precursors into it, indicating cytokinin-stimulated synthesis of the enzyme.

Previous reports of our laboratory in leafy vegetables [15,16,17] have shown that a single BAP spray during the first days after emergence increases total leaf area, fresh weight and dry weight. Cytokinins are a class of plant hormones with a broad range of functions that involve regulation of plant growth and development, physiological processes [18,19] and response of plants to biotic and abiotic stress [20].

The aim of this work was to study the effect of a BAP spray on biomass accumulation and yield in pumpkin plants grown in commercial facilities.

2. MATERIALS AND METHODS

2.1 Plant Material and Experiments

Two experiments were performed:

2.1.1 Experiment 1

The experiment was conducted at the National Institute of Agricultural Technology (INTA), La Consulta Experimental Station, Mendoza, Argentina (33° 43' 59"S, 69° 07' 04" W) from November 11th 2009 to March 23rd 2010 and from November 15th 2011 to April 25th 2012 on a loam-sandy field soil. Water and nutrients were kept at non-limiting levels with an intermittent overhead mist irrigation system, which kept soil water above 50% of maximum soil available water in the first meter of depth. Soil showed a pH = 7.74; electrical conductivity = 2.98 dS/m; N = 630 mg/Kg; P = 5.8 mg/l; K = 19.5 mg/l; organic matter = 0.93% and C/N = 8.51. At the

beginning of the experiments, the experimental field was fertilized with 250 kg ha⁻¹ ammonium phosphate (18-46-0). Weeds were adequately controlled using the herbicide S-Metaloclor. Butternut squash (*Cucurbita moschata*) 'Cuyano INTA' seeds at a 0.33 plants m⁻² were used. Seedlings were sprayed with two concentrations (0 and 100 mg L⁻¹) of BAP (6-benzylaminopurine) (SIGMA EC 214-927-5) two weeks after emergence.

Weather data were recorded from a meteorological station 500 meters from the experimental site. Maximum and minimum air temperature during the experiments was 10.82 and 32.06°C respectively.

2.1.2 Experiment 2

The experiment was conducted at the INTA Balcarce Experimental Station, Buenos Aires, Argentina (37° 45' S, 58° 18' W) during December 2014. Butternut squash 'Cuyano INTA' seeds were germinated and grown in 3-L pots filled with a *Sphagnum maguellanicum*-river waste-perlite (40-40-20, v/v/v) medium [21]. At the beginning of the experiment total porosity (%), air-filled porosity (%), container capacity (%) and bulk density (g cm⁻³) were 63.50, 17.06, 40.06 and 0.35 respectively. Organic matter (%), pH, electric conductivity (dS m⁻¹) and cation exchange capacity were 45.3, 5.2, 0.71 and 58.9 respectively. Seedlings were sprayed with two concentrations (0 and 100 mg L⁻¹) of BAP (6-benzylaminopurine) (SIGMA EC 214-927-5) two weeks after emergence.

A weekly ferti-irrigated control of 1.0: 0.05: 1.0: 0.5 (v/v/v/v) N: P: K: Ca (nitric acid, phosphorus acid, potassium nitrate, and calcium nitrate; Agroquímica Larocca S.R.L., Buenos Aires, Argentina) (150 mg L⁻¹ N) was included through to the overhead irrigation water.

Half hourly averages of the air temperature were measured using a HOBO H08-001-02 data logger (Onset Computer Corporation, MA, USA) protected from direct radiation by aluminum foil shades. Minimum, maximum and mean global solar radiation during the experiment were 13.21°C, 27.71°C and 21.67 MJ m⁻² day⁻¹ respectively.

2.2 Sample and Growth Parameters

Plants for destructive measurements of Experiment 1 were harvested (five per treatment

and block) at emergence and at 30-day intervals during the experiment. Plants were cut at ground level, separated in stems, leaves and petioles oven dried at 80°C for ten days and weighed. Fruit number and fruit weight were recorded at the final harvest.

Plants for Experiment 2 were harvested at emergence (5 plants per treatment) and weekly during the next four weeks. Roots were washed and root, stem, leaf and petioles fresh weights (FW) were recorded. Dry weights (DW) were recorded after drying roots, stems, leaves and petioles to constant weight at 80°C for 96 h.

The number of leaves was recorded in both experiments, and each leaf area was determined using the ImageJ® (Image Processing and Analysis in Java) software.

RGR was calculated as the slope of the regression of the natural logarithm (ln) of the whole plant on a DW basis vs. time (in days), while the rate of leaf area expansion (RLAE) was calculated as the slope of the regression of the ln of total leaf area vs. time (in days). The mean net assimilation rate (NAR) and leaf area partition coefficient (LAP) were calculated as follows:

$$\text{NAR} = (k_w W_0 e^{k_w t}) / (A_0 e^{k_a t})$$

$$\text{LAP} = (k_a A_0 e^{k_a t}) / (k_w W_0 e^{k_w t})$$

where: k_w : RGR (days⁻¹); W_0 : extrapolated value of total dry weight at time zero (g); A_0 : extrapolated value of leaf area at time zero (cm²); k_a : RLAE (days⁻¹); t : time (in days) at the midpoint of the experimental period and e : base of natural logarithms.

Mean leaf area ratio (LAR) was calculated as RGR/ NAR.

The allometric coefficients between root and shoot, and between leaf blades and the petiole-stem fraction were calculated as the slope (β) of the straight-line regression of ln root DW vs. ln shoot DW (ln root DW = $\alpha + \beta$ x ln shoot DW), and between ln leaf blade DW and ln (petiole + stem) DW (ln leaf blade DW = $\alpha + \beta$ x ln petiole-stem DW) respectively.

The harvest index (HI) was calculated as the FW of the harvested fruit as a percentage of the total shoot FW of the plants.

2.3 Statistical Analysis

We used a randomized design with three blocks for Experiment 1 and a complete aleatory design for Experiment 2. Since we found no significant differences in Experiment 1 data between two successive years, we considered them together. Data were subjected to one-way analysis of variance and means were separated by Tukey's test ($P < 0.05$); STATISTICA 8 (StatSoft) software was used. Slopes from straight-line regressions of rate of leaf appearance (RLA), RLAE, RGR, NAR, LAR and allometric values were tested using the SMATR package [22].

3. RESULTS

3.1 Experiment 1

No significant differences were found in Butternut squash yield or its components (individual fruit FW and fruit number) or in HI between plants sprayed or not with a single BAP dose (Table 1).

Although shoot RGR (RGR_s) showed no significant differences, Butternut squash plants sprayed with a single BAP dose two weeks after emergence showed both a lower total leaf area and a lower RLAE as a result of lower individual leaf area and RLA. In contrast, a change was found in Butternut squash crop architecture from larger petioles in BAP-sprayed plants was found (Table 2).

Although Butternut squash plants sprayed with BAP showed no significant differences in photo-

assimilate partitioning between roots and shoots as compared to control plants, they showed a tendency (lower β coefficient in BAP than in control plants) to change photo-assimilate partitioning in favour to shoots (Fig. 1).

3.2 Experiment 2

Results of Experiment 2 were similar to those of Experiment 1. A BAP spray generated Butternut squash plants with lower total and individual leaf area (which decreased RLAE) but higher RLA. The root: shoot ratio was higher in control plants. Although there were no significant differences in RGR, BAP-sprayed plants showed higher NAR and lower LAR and LAP (Table 3).

Allometries between root and shoot showed a proportional photo-assimilate partitioning in both control plants and BAP-sprayed plants; however, in the latter, the DW achieved in shoots was ever higher than in roots (Fig. 2A). When leaves: stems allometries were analysed they showed a higher photo-assimilate partitioning to stems (Fig. 2B).

4. DISCUSSION

Butternut squash yield (on a field area basis) is the result of the fresh weight of each fruit, the number of fruits per plant and the number of plant per unit area. Our results showed that, for the recommended pumpkin plant densities [10,11,12], a single BAP spray two weeks after emergence did not significantly change these traits in control plants (Table 1).

Table 1. The effect of a BAP spray on yield components in Butternut squash. Lower case letters indicate statistically significant differences ($P < .001$). Standard error is also indicated

	Control	BAP
Yield (ton ha ⁻¹)	31.17±2.59 ^a	31.05±2.62 ^a
Fruit fresh weight (kg fruit ⁻¹)	1.23±0.04 ^a	1.27±0.02 ^a
Fruit number (fruit plant ⁻¹)	8.25±0.65 ^a	8.10±0.58 ^a
Harvest Index	0.760±0.012 ^a	0.796±0.006 ^a

Table 2. Changes in total and individual leaf area, petiole length, the relative leaf area expansion (RLAE), the rate of leaf appearance (RLA) and the shoot relative growth rate (RGR_s), in Butternut squash plants sprayed or not with a single BAP dose. Lower case letters indicate statistically significant differences ($P < .001$). Standard error is also indicated

	Control	BAP
Leaf area (cm ² plant ⁻¹)	38,650.87±771.94 ^a	35,957.13±844.99 ^b
Leaf area (cm ² leaf ⁻¹)	246.64±14.72 ^a	172.02±25.21 ^b
Petiole length (cm)	128.30±3.98 ^a	150.24±4.01 ^a
RLAE (cm ² cm ² day ⁻¹)	0.186 ^a	0.173 ^b
RLA (leaves day ⁻¹)	2.940 ^b	3.110 ^a
RGR_s (g g ⁻¹ day ⁻¹)	0.185 ^a	0.180 ^a

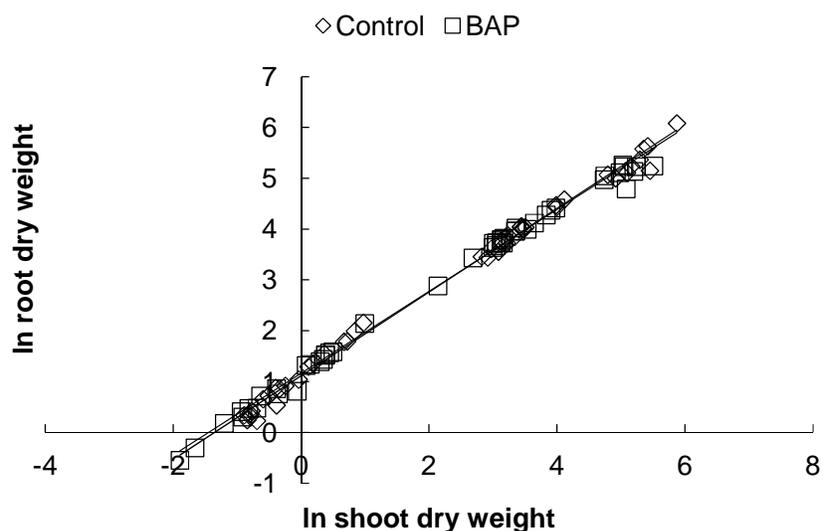


Fig. 1. Changes in the allometric relationships between the roots and shoots (\ln root dry weight = $\alpha + \beta \ln$ shoot dry weight) of Butternut squash plants sprayed or not with a single BAP dose. Linear regression equations are, root dry weight_{Control} = 0.823 shoot dry weight + 1.12 ($r^2 = 0.995$, $P < 0.001$); root dry weight_{BAP} = 0.805 shoot dry weight + 1.61 ($r^2 = 0.995$, $P < 0.001$)

Table 3. Changes in total and individual leaf area, root: shoot ratio, relative leaf area expansion (RLAE), rate of leaf appearance (RLA), relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR) and leaf area partitioning coefficient (BAP) in Butternut squash plants sprayed or not with a single BAP dose. Lower case letters indicate statistically significant differences ($P < .005$). Standard error is also indicated

	Control	BAP
Leaf area (cm ² plant ⁻¹)	851.01±77.84 ^a	723.17±53.11 ^b
Leaf area (cm ² leaf ⁻¹)	93.28±5.22 ^a	81.30±6.89 ^a
Root: shoot ratio	0.234±0.019 ^a	0.158±0.013 ^b
RLAE (cm ² cm ⁻² day ⁻¹)	0.146 ^a	0.132 ^b
RLA (leaf day ⁻¹)	0.241 ^b	0.314 ^a
RGR (g g ⁻¹ day ⁻¹)	0.122 ^a	0.125 ^a
NAR (g cm ² day ⁻¹) × 10 ⁻⁵	44.27 ^b	51.60 ^a
LAR (cm ² g ⁻¹)	274.49 ^a	241.29 ^b
LAP (cm ² day ⁻¹ /g day ⁻¹)	329.83 ^a	255.05 ^b

The determination of RGR [(total biomass (g) per unit of ground area (m²) per unit time (day))] is fundamental to describe squash crop productivity because it provides an estimate of net photosynthesis [9]. When we calculated Butternut squash RGR in our experiments, we found no significant differences between control and BAP-sprayed plants (Tables 2 and 3).

However, the RGR is the product of NAR (net assimilation rate) and LAR (leaf area ratio), where NAR is largely the net result of carbon gain (photosynthesis) and carbon loss (respiration) expressed per unit leaf area. On the other hand, LAR is the ratio between leaf area

and total plant weight and indicates the fraction of total plant weight allocated to the leaves [23]. Our results showed higher NAR (higher photosynthetic capacity) and lower LAR values in BAP-sprayed plants than in control plants (Tables 2 and 3) which explain the lack of significant differences in RGR.

These results are in agreement with previous finding [24], which indicated that the growth-promoting effect of cytokinins on biomass accumulation in the shade ornamental plant *Epipremnum aureum* could be explained by a large increase in NAR, which in turn was associated with a higher net photosynthetic rate.

It has been shown that cytokinins increase the photosynthetic capacity [25,26] and the activity of the main photosynthetic enzyme ribulose-1, 5-bisphosphate carboxylase/oxygenase in rice and *Arabidopsis* [27,28]. In addition, cytokinins increase tetrapyrrole biosynthesis [29] and often increase chlorophyll concentrations.

However, BAP-treated plants expanded a lower both individual and total leaf area per plant. These results would be associated with a change in leaf senescence at the end of experiments but we believe that this was not so in our experiment because RLAE would indicate that BAP-treated plants expanded smaller but more leaves during the experiments (Tables 2 and 3).

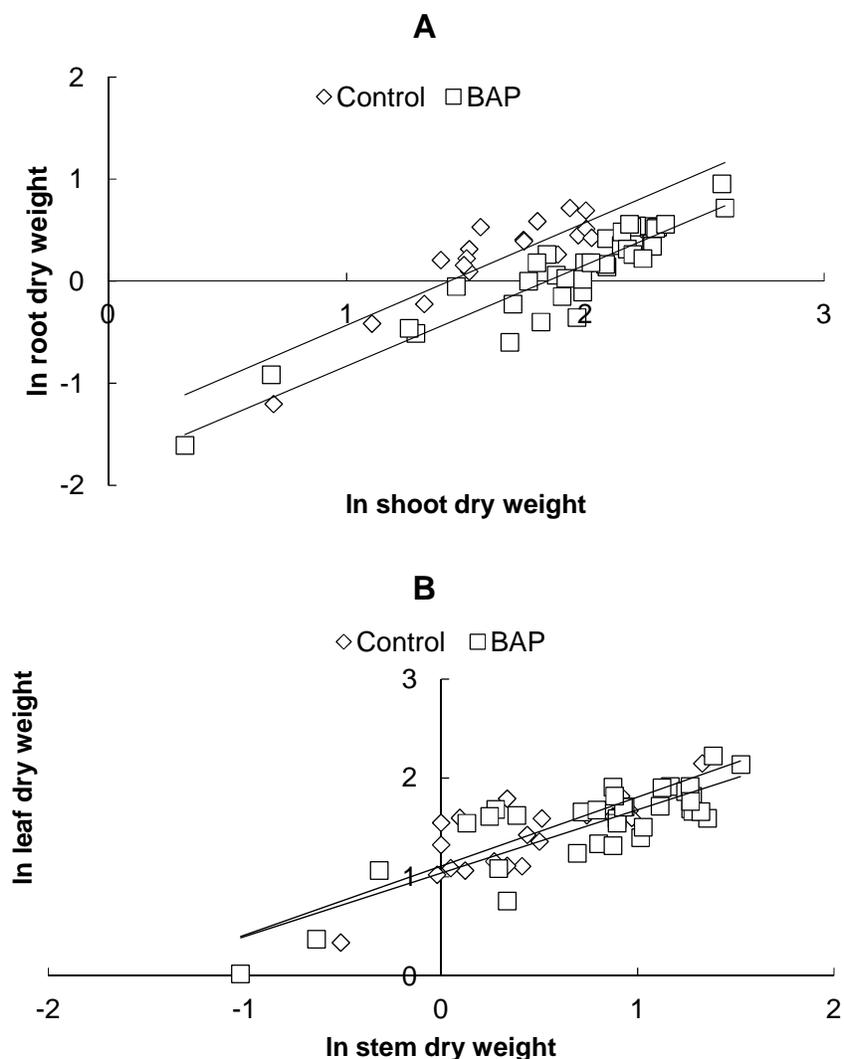


Fig. 2. Changes in the allometric relationships between the roots and shoots ($\ln \text{ root dry weight} = \alpha + \beta \ln \text{ shoot dry weight}$) (A) and between the leaves and stems ($\ln \text{ leaf dry weight} = \alpha + \beta \ln \text{ stem dry weight}$) (B) of Butternut squash plants sprayed or not with a single BAP dose. Linear regression equations are, root dry weight_{Control} = 1.006 shoot dry weight - 1.44 ($r^2 = 0.745$, $P < 0.001$); root dry weight_{BAP} = 0.992 shoot dry weight - 1.82 ($r^2 = 0.850$, $P < 0.001$); leaves dry weight_{Control} = 0.697 stem dry weight + 1.11 ($r^2 = 0.631$, $P < 0.001$); leaves dry weight_{BAP} = 0.639 stem dry weight + 1.04 ($r^2 = 0.695$, $P < 0.001$)

Squash leaf production depends upon the rate of new leaf initiation, the degree of branching or number of shoot meristems, and the rate of leaf expansion and final leaf size [9]. In our experiments, Butternut squash plants developed two to three lateral branches from lower nodes along the main stem so that new growth arose from both the main stem and primary branches; this was followed by formation of the secondary lateral branches arising from primary branches (data not shown).

Plant development and shoot growth depend on the ability of the shoot apical meristem to grow and to initiate new organs. The co-ordination of growth in such a dynamic structure requires extensive short- and long-distance intercellular signalling. Leaves are formed from an initial group of cells within the meristem; one of the earliest markers of leaf initiation is the downregulation of *KNOX* genes in these cells [30]. Genetic analyses have demonstrated that a high cytokinin: low gibberellin ratio is important for *KNOX* gene function [31]. Plants that over-express *KNOX* genes seems to have increased cytokinin levels [32].

Cytokinins have a strong influence on many aspects of shoot development and metabolism, including branching [33] and leaf initiation [34]. However, in squash, the concentrations of the physiologically active cytokinin bases, ribosides and nucleotides, as well as those of the cis-isomers of zeatin derivatives, decrease between the first and fifth weeks of cultivation under controlled light conditions [35].

The final size of plant organs such as leaves is tightly controlled by environmental and genetic factors that spatially and temporally coordinate cell expansion and cell cycle activity [36,37]. It has been suggested that cytokinins play a role in cellular differentiation and in the maintenance of vegetative apex [38] and that they can promote leaf unfolding and expansion in whole plants in several species [39]. Any perturbation in one of these processes by modifications in the expression of specific genes might therefore affect the final leaf area [40,41].

The most usual result in BAP-sprayed plants is an increase in RLA due a change in shoot apical meristem growth. The possibility that the rate at which leaves appear in a vegetative meristem could be regulated by exogenous application of BAP has been explored only recently [42,43,24,17]. Some authors have claimed that the plastochron (i.e., the time between

successive leaf initiation events) may be altered in transgenic plants with reduced cytokinin levels [44,45]. However, the possibility that exogenous application of a cytokinin may affect the plastochron has attracted little attention. Cytokinins positively regulate organ size, while extending the length of the phyllochron (i.e., delaying the production of new leaves), thus providing a compensatory mechanism that links the rate at which leaves are produced to the final leaf size. Such a mechanism would mean that the overall rate of production of biomass would remain relatively constant. This would be achieved by producing either numerous small organs, or fewer (but larger) organs [46].

On the other hand, in the present study, we found a significant change in photo-assimilate partitioning to favour shoots in BAP-sprayed plants, indicated by the LAP coefficients, the root: shoot ratio (Table 3) and the plant allometries between the ln of root dry weight vs. ln of shoot dry weight (Figs. 1 and 2). These results are in agreement with previous reports from our laboratory in vegetables [47,16,17] and ornamental plants [43,24,48,49].

Both leaf initiation and expansion needs for photo-assimilates [50,51]. A decrease in the plastochron needs an increase in apex size [52,53] which is, in turn, related to the rate of leaf appearance as has been shown in experiments with cytokinin deficient *Arabidopsis* mutants [54,55]. Because pumpkin showed relatively high mean leaf size (Tables 1 and 2) [56], an insufficient supply of sugars would limit leaf expansion more than leaf initiation [57]. Milla et al. [58] showed that large leaves tend to require higher biomass investments per unit leaf area than small leaves. On the other hand, some researchers have found an interaction between sugars and cytokinins [59]. Plant allometries between ln shoots dry weight vs. leaf dry weight showed a higher photo-assimilate partitioning to leaves in BAP-sprayed plants (Fig. 2B) in agreement with previous finding [24].

During initial development, the normal growth rate of a Cucurbit leaf is maintained by importing soluble carbohydrates in the phloem from mature leaves. As leaf development progresses, the growth rate declines but the photosynthetic capacity of the young leaf continues to increase thereby diminishing its dependence on the older leaves for additional carbohydrate supplies [60]. BAP-sprayed plants would increase the supply of photo-assimilates through its activity on vascular development [61,62].

Hormones show a dual-action mechanism; at relative low concentrations, they would stimulate a process whereas and at relative high concentrations, they would inhibit the same process [63,15]. Although this hypothesis cannot be excluded, further experiments are required to validate it.

The strategies to increasing yield in squash include producing seedless fruit and exploiting heterosis as a means for increasing use of pumpkin F₁ hybrids, the use of heterosis as a means for increasing productivity within the genus *Cucurbita* has been recently validated [64,65]. Bennett et al. [66] showed that shoot architecture could have a substantial impact on the partitioning of reserves between vegetative and reproductive tissues and could be an important trait for selection in rapid phenotyping screens to optimize crop performance.

To understand the mechanisms associated with the morphological changes related to a BAP spray and their importance in population architecture would be a key matter for a future breeding program.

5. CONCLUSIONS

To understand the mechanisms associated with the morphological changes related to a BAP spray and their importance in population architecture would be a key matter for a future breeding program. From the growers' point of view, the use of a BAP spray would not increase yield at the current plant densities suggested. However, the changes in crop architecture from lower individual leaf size but higher photosynthetic capacity would allow increasing the plant densities suggested, after two decades. However, the validation of this hypothesis goes beyond the present experimental design and is the matter of further experiments, which are already in progress.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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