

Effects of autoclaving on the physiological action of paclobutrazol

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ABSTRACT

Besides being employed as an efficient plant growth retardant for field, garden and potted plants, paclobutrazol (PBZ) is also used in laboratory experiments, mainly in aseptic cultures, both in autoclaved and non-autoclaved form. Therefore it is not known if autoclaving can partial or completely inactivate the product, thus decreasing its efficacy. Thus a simple experiment was carried out to assess to what extent the autoclaving process can affect some growth components and dry mass accumulation and partition in sunflower seedlings, by employing the techniques of Plant Growth Analysis. Autoclaving did not modify either qualitative or quantitatively any of the plant responses to PBZ as well their reversions by gibberellic acid.

Keywords: Autoclaving; Dry Mass Accumulation; Gibberilins; Growth; Paclobutrazol; Sunflower

1. INTRODUCTION

Paclobutrazol [PBZ; common names: Bonzi, Clipper-S, Clipper-T, Cultar, PP333; chemical name: IUPAC: (2*RS*,3*RS*)-1-4-(chlorophenyl)-4,4-dimethyl-2-(1*H*,1,2,4-triazol-1-yl)pentan-3-ol] is an efficient plant growth retardant listed for usage by the Plant Growth Regulation Society of America [1]. Its optical enantiomer 2*S*,3*S* displays a pronounced plant growth regulatory activity, whereas the 2*R*,3*R* enantiomer is more active in the inhibition of sterol biosynthesis, exhibiting fungicidal properties. The growth retarding form of PBZ (*S*-enantiomer) keeps certain structural similarities with *ent*-kaurene and *ent*-kaurenol, key compounds in the pathway to gibberellins (GAs) biosynthesis, and hence may inhibit cytochrome P450 monooxygenases, impairing the oxidation of *ent*-kaurene to *ent*-kaurenoic acid [2,3]. As a result PBZ can cause a substantial depletion in the lev-

els of active GAs throughout the plant [3], leading to a kind of dwarfism [1]. Some effects of PBZ on plants are: development of dark green color in leaves [2]; stimulation of flowering, likely as a result of a decrease in vegetative growth [4,5]; hastened leaf fall in autumn and emergence delay in spring in deciduous plants [2]; yield increase in apples [6], *Jathropa curcas* [7] and other cultivated species.

Water solubility of PBZ is very poor, about 0.12 mM (35 mg·dm⁻³; [1,2]), the compound being mostly immobile in the phloem sieve tube elements. Due to that when sprayed in the plants its action is much localized. A more uniform distribution is seen when it is provided by trunk injection or soil application, because in this case the product is transported via xylem vessels [8]. This low solubility has brought about some troubles especially when the compound is used under laboratory conditions. For instance in order to achieve reproducible results on the inhibition of seed germination of Townsville stylo (*Stylosanthes humilis*) PBZ had to be autoclaved [9]. The autoclaved product is also largely employed in aseptic culture experiments [10-12], although the non-autoclaved form is identically used [13-15]. Sometimes the condition of the PBZ (autoclaved or non-autoclaved) employed in the experiments is not even mentioned [16]. Since PBZ is “stable at all temperatures up to 50°C for at least six months” [1,2] and that during the autoclaving process temperature as high as 120°C is achieved, it is likely that heating leads to degradation of the compound, rendering PBZ completely or partially inactive. As a consequence the effective amount of PBZ that elicits a physiological action is not known when the product is autoclaved. In order to throw some light to this problem a simple assay was designed as to compare the effects of autoclaved and non-autoclaved PBZ on the growth and dry mass accumulation of seedlings of sunflower (*Helianthus annuus* L.).

2. MATERIALS AND METHODS

Seeds of sunflower ‘Agará 4’ plants were sown in 400

cm³ plastic pots containing a 2:1:1 mixture of red-yellow latosol soil, sand and cattle manure in a greenhouse in Viçosa (20°45' S, 42°15' W), Minas Gerais state, Brazil. Upon seedling emergence, soil was fertilized with 0.2 g of NPK (25-5-20). When *ca* 7 - 10 cm high, seedlings were thinned to two plants per pot. On the occasion 5 cm³ PBZ (10% a.i.; Wisser Importer, SP, Brazil) suspension (containing 2.5 or 5 mg) were distributed with a pipette on the soil surface of each pot (day 0, **Figure 1** and **Table 1**).

Autoclaving of PBZ was accomplished in an autoclave (Sercom, Model HA, SP, Brazil), for 20 min, under 121°C and a pressure 120 kPa. Nine and 16 days after PBZ application, seedling apices were sprayed until

complete wetness with a 0.1 mM gibberellic acid (GA₃; SIGMA, MI, USA) solution also containing 0.05% Tween 80. Stem length, as measured from soil surface to the uppermost visible node, was registered at each other day. At the end of the experiment the plants were harvested, their roots were washed and leaves (unfolded \geq 1.5 cm) and internode number counted. Following separation roots stems and leaves were oven-dried at 70°C for about 72 h, and afterwards their dry mass determined. Organ lengths and dry masses constituted thus the primary data upon which the Growth Analysis techniques were used according to Richards [17] and Hunt *et al.* [18].

Statistical design was based on a completely random-

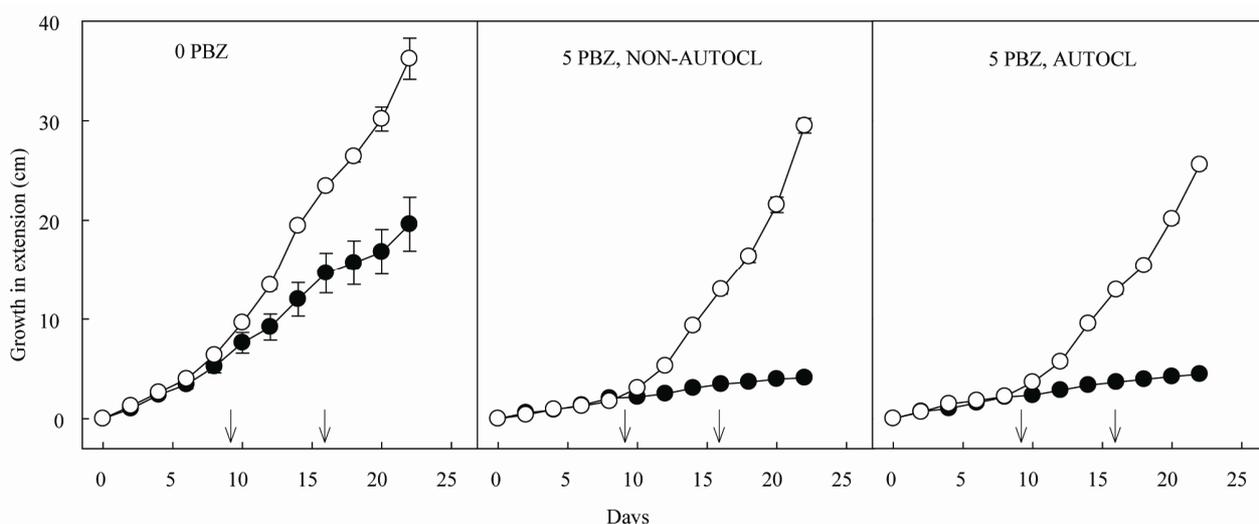


Figure 1. Growth increase in stem length of sunflower seedlings as affected by autoclaved (AUTOCL)- and non-autoclaved (NON-AUTOCL)-PBZ applied in the soil surface at the dosage 5.0 mg per pot at the day 0. At days 9 and 16 following PBZ application, GA₃ (0.1 mM) was sprayed at the shoot apex of the plants, as indicated by the arrows. The pattern of growth curves in response to 2.5 mg PBZ per pot was exactly the same as shown in the figure, except for the magnitude of the values. Full circles: -GA₃; Hollow circles: GA₃-treated. Each point and bars represent means \pm SE of 10 plants.

Table 1. Increase in stem (STM) length, mean internode (INT) length and stem growth rate of sunflower plants before (0 - 9th day) and after (10th - 22nd day) the first plant spraying with GA₃. In this table and **Tables 2** and **3** numbers following PBZ represents its doses (mg) applied in each pot. GA₃ was sprayed at the concentration 0.1 mM. CTL-control. (Means to be compared within columns; values followed by the same letter do not differ significantly at 5% by Tukey test. Data analysis by Scott-Knott test also showed that the effects of autoclaved and non-autoclaved PBZ were similar).

Treatment	STM length	INT length	STM growth rate (cm·day ⁻¹)	
	(cm)	(cm)	(0 - 9th day)	(10 - 22nd day)
CTL	19.6 \pm 2.7 c	2.19 \pm 0.30 c	0.65 \pm 0.08 a	1.00 \pm 0.15 b
CTL + GA ₃	36.2 \pm 2.1 a	3.3 \pm 0.17 a	0.80 \pm 0.04 a	2.21 \pm 0.14 a
Non-autoclaved:				
PBZ 2.5	5.0 \pm 0.3 d	1.14 \pm 0.06 d	0.31 \pm 0.03 bc	0.15 \pm 0.01 c
PBZ 5.0	4.1 \pm 0.3 d	0.93 \pm 0.04 d	0.26 \pm 0.02 bc	0.16 \pm 0.01 c
PBZ 2.5 + GA ₃	28.1 \pm 0.7 b	3.05 \pm 0.14 b	0.28 \pm 0.02 bc	2.06 \pm 0.04 a
PBZ 5.0 + GA ₃	29.5 \pm 0.8 b	3.09 \pm 0.05 b	0.22 \pm 0.02 c	2.20 \pm 0.07 a
Autoclaved:				
PBZ 2.5	5.4 \pm 0.3 d	1.17 \pm 0.04 d	0.33 \pm 0.01 bc	0.21 \pm 0.01 c
PBZ 5.0	4.5 \pm 0.2 d	1.00 \pm 0.06 d	0.27 \pm 0.02 bc	0.18 \pm 0.01 c
PBZ 2.5 + GA ₃	29.5 \pm 2.2 b	3.15 \pm 0.08 ab	0.39 \pm 0.03 b	2.01 \pm 0.15 a
PBZ 5.0 + GA ₃	25.6 \pm 0.4 b	2.78 \pm 0.11 bc	0.28 \pm 0.02 bc	1.83 \pm 0.03 a

ized distribution with five replicates, each one constituted of a pot with two plants, per treatment. The Tukey mean separation test, at 5% level, was employed to detect differences amongst treatments. As Scott-Knott test clusters means without superimposing their significances, it was employed further as to subsidize the Tukey's analyses. In this way probable differences and similarities among the treatments could be further confirmed.

3. RESULTS AND DISCUSSION

The first signs of PBZ action were observed by the development of a dark-green color in growing leaves [2] 3 - 4 days after application of the product to the soil. The new appearing leaves then became smaller [19], exhibiting a thicker and wrinkled aspect [20]. Thereafter the growth rate of PBZ-treated plants decreased substantially (**Figure 1, Table 1**) and the apical internodes and leaves started displaying a rosette form. This dwarfism syndrome was similarly exhibited by autoclaved and non-autoclaved PBZ-treated plants, with no quantitative differences between the plants of the two treatments (Tukey and Scott-Knott tests, 5% level). Each one of those symptoms was properly reverted by GA₃, the responses also not being affected by the autoclaving of PBZ.

Stem growth pattern followed the well-characterized sigmoidal logistic model [17,18]. As **Figure 1** and **Table 1** show stem length resulted much longer in the control and in all GA₃-treated plants than in PBZ-inhibited plants. Again, differences in stem length in response to autoclaved and non-autoclaved PBZ were too small to have assumed any statistical significance. A dramatic change in the growth pattern of PBZ-treated plants was observed following GA₃ application (**Figure 1**). Growth rates increased substantially, sometimes higher than 10-fold (**Table 1**); in this way, at the end of the experiment the stem length of these plants surpassed that of the control non-treated plants.

Leaf and internode number (about the half of the leaf number, within a range 10.0 - 11.6 internodes per plant, not shown) were not affected by any of the treatments, showing that in sunflower they constitute highly conserved traits. Hence the much reduced stem length caused by PBZ (**Figure 1, Table 1**), was a consequence of the very shorter internodes, an effect also reverted by GA₃ notwithstanding whether PBZ was autoclaved or not (**Table 1**).

Total dry mass accumulated per plant did not respond to any of the treatment, autoclaved PBZ included (**Figure 2, Table 2**). In this context, in some plant species GAs affect positively the net CO₂ assimilation (*A*) whilst in others it is not affected [21]. Sunflower thus, seems to belong to the latter group. This fact must be a consequence of a similar *A* displayed by PBZ-treated and non-treated sunflower seedlings [22]. Similarly dry mass allocation to leaves and to the stem-root system was not affected either by PBZ or GA₃ (**Figure 2, Table 2**). The regulators, however, affected the dry mass partition within the stem-root system (see below).

Since leaf dry mass per plant was not affected by the regulators which also did not affect leaf number, the mean individual leaf dry mass was also kept similar amongst all the treatments (range 79.0 - 99.0 mg, not shown). As a consequence of the similar total dry mass per plant and also of a similar leaf dry mass per plant, leaf mass ratio (LMR), the ratio between leaf to total plant dry mass [23], did not show any significant difference among the treatments (**Table 3**). Though the mean individual leaf dry mass did not vary among the treatments, they profoundly affected leaf size as mentioned above. As described earlier leaf size was drastically reduced by PBZ, as also found in *Zinnia* and *Geranium* [24]. In order to hold the same dry mass in a much reduced area PBZ-treated leaves have to be thicker, a point examined in a side experiment with the third uppermost leaves in the stem (showing symptoms of PBZ action).

Table 2. Dry mass (DRM) of roots (ROT), stem (STM), leaves (LFY) of sunflower plants. Also shown are stem plus root (STM + ROT), total (TOT) dry mass per plant, and mean internode (INT) dry mass. (Means to be compared within columns; values followed by the same letter do not differ significantly at 5% level by Tukey test. Data analysis by Scott-Knott test also showed that the effects of autoclaved and non-autoclaved PBZ were similar).

Treatment	ROT (g)	STM (g)	LFY (g)	STM+ROT (g)	TOT (g)	INT (mg)
CTL	0.56 ± 0.07 abcd	0.85 ± 0.14 ab	0.75 ± 0.04 a	1.40 ± 0.16 a	2.15 ± 0.16 a	81 ± 15 abc
CTL + GA ₃	0.38 ± 0.03 d	1.05 ± 0.11 a	0.77 ± 0.03 a	1.43 ± 0.13 a	2.20 ± 0.17 a	100 ± 10 a
Non-autoclaved:						
PBZ 2.5	0.70 ± 0.03 ab	0.64 ± 0.04 bc	0.98 ± 0.06 a	1.34 ± 0.06 a	2.31 ± 0.23 a	56 ± 4 bcd
PBZ 5.0	0.70 ± 0.08 ab	0.50 ± 0.06 c	0.81 ± 0.05 a	1.20 ± 0.13 a	2.01 ± 0.17 a	46 ± 5 d
PBZ 2.5 + GA ₃	0.49 ± 0.06 bcd	1.06 ± 0.06 a	0.80 ± 0.03 a	1.55 ± 0.10 a	2.36 ± 0.13 a	92 ± 7 a
PBZ 5.0 + GA ₃	0.51 ± 0.04 bcd	1.05 ± 0.02 a	0.91 ± 0.03 a	1.56 ± 0.03 a	2.47 ± 0.02 a	94 ± 2 a
Autoclaved:						
PBZ 2.5	0.80 ± 0.06 a	0.56 ± 0.04 bc	0.74 ± 0.09 a	1.36 ± 0.09 a	2.10 ± 0.17 a	57 ± 3 bcd
PBZ 5.0	0.64 ± 0.05 abc	0.53 ± 0.05 bc	0.87 ± 0.04 a	1.17 ± 0.07 a	2.04 ± 0.11 a	51 ± 5 cd
PBZ 2.5 + GA ₃	0.47 ± 0.04 bcd	1.02 ± 0.08 a	0.91 ± 0.07 a	1.49 ± 0.11 a	2.40 ± 0.17 a	90 ± 4 a
PBZ 5.0 + GA ₃	0.40 ± 0.03 cd	0.99 ± 0.03 a	0.88 ± 0.07 a	1.39 ± 0.06 a	2.27 ± 0.12 a	87 ± 4 ab

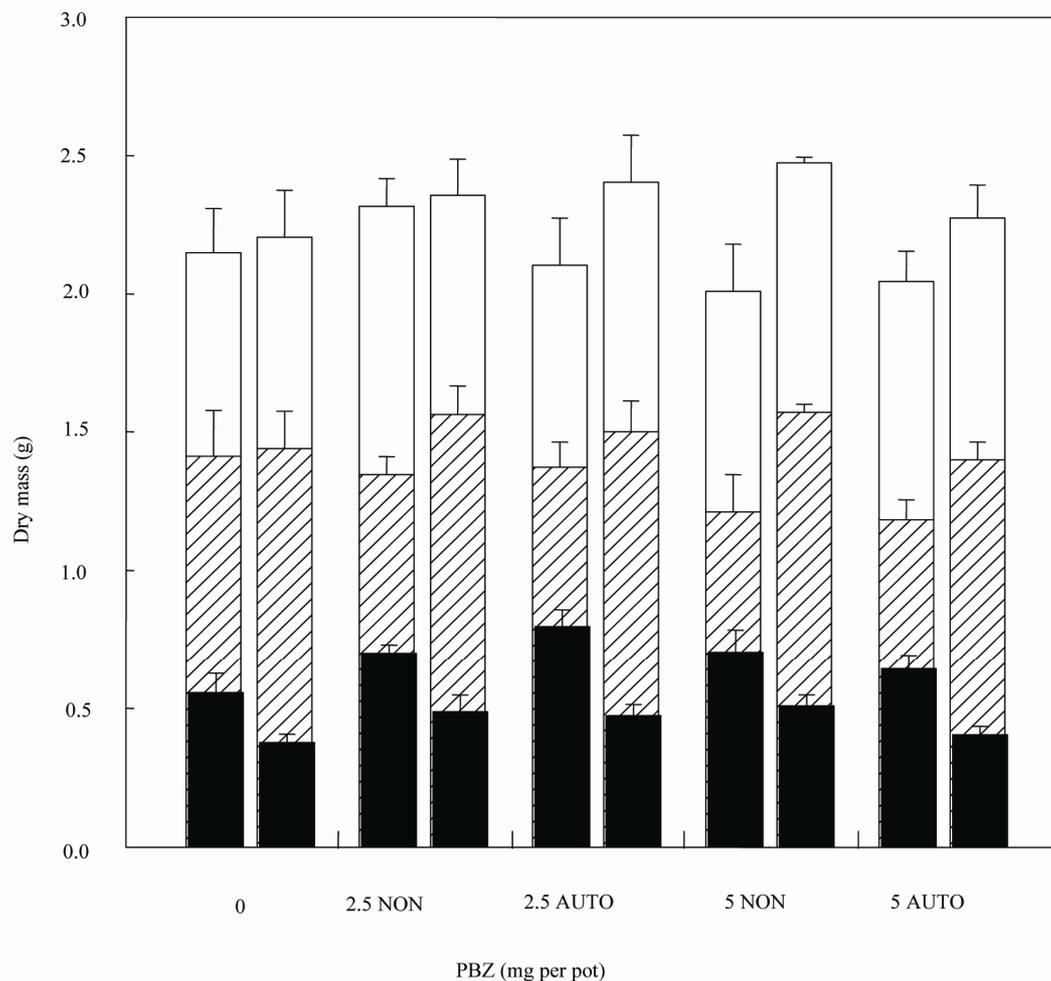


Figure 2. Dry mass accumulation by sunflower seedlings and partitioning among leaves (hollow rectangles), stem (hatched rectangles) and roots (full rectangles). Total plant dry mass, total leaf dry mass and stem plus root dry mass did not show any statistical significance among the treatments; differences were exhibited between shoot and root dry mass of plants of the several treatments (Tukey's and Scott-Knott's tests, 5% level). Figures represent the amounts of PBZ in mg applied to each pot. For each pair of data of control (0), autoclaved (AUTO)- and non-autoclaved (NON)-PBZ treatments, the 1st element of the pair refers to -GA₃ treatment and the second one to +GA₃ treatment. Bars represent \pm SE around means of 10 plants.

Specific leaf area (SLA, obtained by dividing the area by leaf dry mass [23]), was about 31.7 in control leaves, 26.5 - 28.8 in PBZ-treated leaves and 36.9 - 43.0 m²·kg⁻¹ in GA₃-treated leaves. PBZ (autoclaved and non-autoclaved) thus induced the formation of small and thicker leaves, an effect promptly reverted by GA₃.

Shoot-root system dry mass as a whole did not respond to any of the regulators employed (**Figure 2, Table 2**). Since total dry mass per plant was not affected as well, the ratio stem-root to total plant dry mass (range 0.57 - 0.66) was equally not affected by them (**Table 3**). Within that system itself each one stem and root dry mass was, nevertheless, greatly affected (**Figure 2, Table 3**). As shown in **Figure 1**, stem expansion was highly responsive to the regulators. Hence the system

stem-root seemed to be the main target for the action of PBZ and GA₃. When dry mass of stems and roots were considered separately they showed a large variation in response to treatments (**Figure 2, Tables 2 and 3**), PBZ (autoclaved and non-autoclaved) favoring dry mass allocation to roots and GA₃ to stems. Hence the ratio root to total plant dry mass (root mass ratio, RMR; [22]) was larger in PBZ-treated plant whereas stem to total plant dry mass (stem mass ratio, SMR) was larger in all (including the reversion of PBZ effects) GA₃-treated plants (**Table 3**). In agreement with these observations, the root biomass accumulation was also increased in uniconazol- and PBZ-treated plants of *Arabidopsis* [25]. Moreover these data are in close accordance with recent genetic studies which revealed good correlation between a low

Table 3. Some allometric relationships in sunflower plants as affected by PBZ and GA₃. Root mass ratio (RMR, fraction of root mass to total plant dry mass); stem mass ratio (SMR, fraction of stem mass to total plant dry mass), leaf mass ratio (LMR, fraction of leaf mass to total plant dry mass); STM + ROT:TOT (stem plus root to total plant dry mass); root to shoot dry mass (ROT:SHT); root to stem plus root dry mass (ROT:STM + ROT); and dry mass distribution over the stem length unit (DRM/LNT). (Means to be compared within the columns. Values followed by the same letter do not differ significantly at 5% level by Tukey test. Data analysis by Scott-Knott test also showed that the effects of autoclaved and non-autoclaved PBZ were similar).

Treatment	RMR	SMR	LMR	STM + ROT: TOT	ROT:SHT	ROT: STM + ROT	STM DRM/ LNT (g·m ⁻¹)
CTL	0.26 ± 0.03 cd	0.39 ± 0.05 a	0.35 ± 0.03 a	0.64 ± 0.07 a	0.35 ± 0.05 cd	0.41 ± 0.05 b	3.6 ± 0.8 b
CTL + GA ₃	0.17 ± 0.01 e	0.47 ± 0.02 a	0.35 ± 0.02 a	0.64 ± 0.04 a	0.21 ± 0.01 e	0.27 ± 0.01 c	2.6 ± 0.2 b
Non-autoclaved:							
PBZ 2.5	0.30 ± 0.01 bc	0.27 ± 0.01 b	0.42 ± 0.02 a	0.58 ± 0.04 a	0.44 ± 0.03 bc	0.53 ± 0.02 a	4.9 ± 0.4 a
PBZ 5.0	0.35 ± 0.02 ab	0.24 ± 0.01 b	0.41 ± 0.02 a	0.59 ± 0.05 a	0.54 ± 0.03 ab	0.59 ± 0.08 a	4.9 ± 0.3 a
PBZ 2.5 + GA ₃	0.20 ± 0.01 de	0.45 ± 0.01 a	0.34 ± 0.01 a	0.66 ± 0.02 a	0.26 ± 0.02 de	0.31 ± 0.02 bc	3.0 ± 0.1 b
PBZ 5.0 + GA ₃	0.21 ± 0.02 de	0.42 ± 0.01 a	0.37 ± 0.01 a	0.63 ± 0.02 a	0.26 ± 0.03 de	0.33 ± 0.02 bc	3.0 ± 0.1 b
Autoclaved:							
PBZ 2.5	0.38 ± 0.02 a	0.27 ± 0.01 b	0.35 ± 0.02 a	0.65 ± 0.05 a	0.62 ± 0.05 a	0.59 ± 0.02 a	4.8 ± 0.2 a
PBZ 5.0	0.31 ± 0.03 abc	0.26 ± 0.02 b	0.43 ± 0.01 a	0.57 ± 0.02 a	0.46 ± 0.03 bc	0.55 ± 0.03 a	5.0 ± 0.4 a
PBZ 2.5 + GA ₃	0.20 ± 0.01 de	0.42 ± 0.01 a	0.38 ± 0.01 a	0.62 ± 0.02 a	0.25 ± 0.01 de	0.32 ± 0.01 bc	2.9 ± 0.1 b
PBZ 5.0 + GA ₃	0.18 ± 0.01 e	0.43 ± 0.01 a	0.39 ± 0.01 a	0.61 ± 0.03 a	0.22 ± 0.01 de	0.29 ± 0.01 c	3.1 ± 0.1 b

GA regime and biomass accumulated in the roots [26].

A pattern emerges as to explain the results on dry mass distribution in sunflower plants when the relationships between length and dry mass of the stem as affected by PBZ and GA₃ are taken into account. It is well known that GA₃ stimulates stem growth towards the shoot main axis direction which is translated into internode expansion (**Figure 1, Table 1**). PBZ, on the other hand, inhibits stem elongation leading to much smaller cells. GA₃-treated stems accumulate much more dry mass just because they are much longer than PBZ-treated stems (**Figure 2, Tables 1 and 2**). By taking into account the dry mass distribution over the stem length unit, it is seen that both autoclaved and non-autoclaved PBZ-treated stems similarly “concentrate” more dry mass than GA₃-treated stems (**Table 3**), being thus “denser”. It is deduced that dry mass accumulation by individual cells of PBZ-treated plants may approach or even reach its potential, what did not absolutely occur in GA₃-treated stems.

Given that dry mass accumulation by the stem-root system does not vary with treatments (**Figure 2, Table 2**) a model can then be proposed that fits to the above facts. In GA₃-treated plants stem elongates excessively but dry mass accumulation (about 60% of dry mass accumulated by PBZ-treated stems, on a length unit basis, *i.e.*, far from its maximum potential, see **Table 3**) does not keep pace with that process, leading to the production of narrower and elongated stems. Nevertheless, due to this high elongation (**Figure 1, Table 1**), stem constitutes the main sink of the stem-root system and thus in GA₃-treated plants little dry mass remains to be allocated to the roots (**Table 3**). A low root to shoot ratio (or root to stem plus root) thus results (**Table 3**). In PBZ-treated plants, stems are very short and although they accumulate more dry mass per volume unit (“denser” cells, **Table 3**), they are too small to constitute a strong assimilate sink. The surplus of assimilates produced by the plant is then directed towards the roots, what explains the high root to shoot (or root to stem plus root) ratio displayed by PBZ-treated plants (**Table 3**). Curiously, these calculations led to similar results as the ones obtained in [21] with tomato using much more sophisticated techniques. By working with the A70 (moderately deficient) and W335 (extremely deficient) GA slow-growing mutants, those authors showed that the shoot constituted the main target for GA action. Accordingly, as growth in shoot elongation was much smaller in GA-deficient tomato plants much more assimilate was left to be partitioned into the roots as compared with the wild type plants. Summarizing, this dry mass accumulation pattern was due to stem growth as affected by GAs and not to be regulator by itself. Since total dry mass in sunflower plants did not respond to the regulators herein employed being kept more or less constant (**Table 2**), a good theoretical exercise would be to forecast where the assimilates would come from in case the cells affected by GA₃ would accumulated dry mass to their full potential.

In summary when investigating the effects of autoclaving in PBZ physiological action on the growth and dry mass partition in sunflower seedlings, no significant difference in the inhibitory effects of autoclaved and non-autoclaved form was found and in the reversion of those effects by GA₃, as well. Hence, the amounts (levels) of autoclaved PBZ employed in aseptic cultures or used for other purposes in laboratory or field conditions could be considered as the real one and equivalent to the amounts of the non-autoclaved form. This has already been put forward in [10], without showing any evidence, however. As expected the main effects of PBZ and GA₃ were observed in shortening and elongation of seedlings stems, respectively. The body of evidences leads to the

conclusion that GA deficiency caused by PBZ (or in GA-deficient mutants) resulted in a pronounced stem growth decrease and hence as stem-root system dry mass did not vary to a large extent, assimilates are directed towards the roots. Unequivocally, in the present investigation, not at all affected by both GA₃ and PBZ (autoclaved and non-autoclaved) were mean leaf and internode number, total plant dry mass, leaf dry mass per plant, and the stem-root system dry mass per plant as a whole.

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List of abbreviations:

CLT: control; DRM: dry mass; GA, GA₃: gibberellins, gibberellic acid; LFY, leaf; LNT: length; PBZ: pa-

clobutrazol; ROT: root; SHT: shoot; STM: stem; TOT: total.