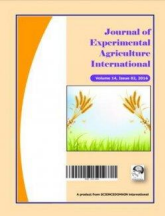

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Exogenous BAP Spray Applications against to Abiotic Stress Related by Root Restrictions in Spinach

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ADB, JDM and EG designed the study and wrote the manuscript. Authors JG and VF recorded data, performed the statistical analysis and managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Previous reports showed that the abiotic stress related to the pre-transplant plug cell volume during nursery decreases both root and shoot growth at the transplant stage and limit final yield to leafy green vegetables. The cytokinin function has been linked to different abiotic stresses including plug cell volume during nursery, which explain that a single early benzyl amino purine (BAP) spray can override root restriction effects. Since transplanting has almost replaced direct seeding, the objective of this new report was to analyze spinach growth changes of different root restrictions degree by direct-seeded or the use of different plug cell volumes but including the use of the hormonal regulator BAP as an abiotic stress alleviator at different times. Our results showed that higher yield has been related to leaf area expansion (estimated through RLA, RLAE and individual

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leaf size), photo assimilate acquisition (estimated through RGR, NAR and SLA), and photo assimilate partition (estimate through root-shoot allometries). All traits can be modified by root restriction during nursery and a single BAP spray. On the other hand, a direct relationship between RLAE, RLA, RGR, NAR, glucose content and root dry weight was found. However, the precise quantitative response is related to BAP application time as well. In summary, plug cell volume can be considered as an abiotic stress, which decreases spinach yield. Shoot-biomass accumulation can be optimized through direct-seeded, increased plug cell volume or applied a single BAP spray in plug-grown plants. However, a precise BAP application time and spinach response relationship can be shown.

Keywords: Abiotic stress; exogenous cytokinin; nursery; yield.

1. INTRODUCTION

The increased use of plug trays for nursery growth is supported by faster and a more uniform growth after transplanting, earlier and more uniform yields, increased production per unit area and time and better use of seed and space for most vegetables. However, pre-transplant plug cell volume creates an abiotic stress named "root restriction syndrome", which decreases both root and shoot growth at the transplant stage and limit final yield to leafy green vegetables [1], including spinach [2].

The growth rates and final size of plant organs are determined by both genetic constraints and environmental factors that must spatially and temporally coordinate cell expansion and cell cycle activity [3,4]. Abiotic stress is defined as environmental conditions that reduce growth and yield below optimum levels, but investigating how abiotic stresses affect plant growth and development at the physiological, biochemical, and molecular levels is critical to increasing the productivity of crops [5].

Hormones, such as cytokinin, plays a role in the response to many environmental signals [6] and are also important regulators of plant responses to abiotic stress [7,8] via a complex network of cytokinin signaling [9,10]. In the same way, Çakmakçı et al. [11] showed that spinach seedlings growth might be enhanced by phytohormone producing bacteria. On the other hand, Zwack and Rashotte [12] indicate that multiple factors influence how cytokinin treatment affects stress signaling and that the spatial, temporal, and developmental context may be important factors in the downstream stress response.

Previous reports from our laboratory in ornamentals [13,14,15] and vegetables [16,17,1,2,18,19] showed that a single early benzyl amino purine (BAP) spray can override root restriction effects by increasing leaf area and biomass shoot accumulation. BAP effects on lettuce and celery are especially sensitive to hormone concentration and application time [1]; two key traits for commercial suggestions because the composition and concentration of cytokinins in the site of action might be quite different from those in the site of application [20].

Spinach (*Spinacia oleracea* L.) is an important leafy green vegetable with a high biological value, which contains large quantities of bioactive compounds especially when fresh, steamed, or quickly boiled. On the other hand, it is one of the best-sold frozen vegetables [21]. Spinach is unique among vegetable crops because of its extremely high yield production in a relatively short period of time [22]. It is a cold-adapted plant, which has been cropped mainly during winter but, nowadays, a higher demand on summer months run the risk of different abiotic stresses.

Since transplanting has almost replaced direct seeding, the objective of this new report was to analyze spinach growth changes of different root restrictions degree by direct-seeded or the use of different plug cell volumes but including the use of the hormonal regulator BAP as an abiotic stress alleviator at different times.

2. MATERIALS AND METHODS

2.1 Plant Material and Treatments

The experiment was carried out in a greenhouse at the Faculty of Agronomy, University of Buenos Aires, Argentina (34°28'S) on Toucan RZ (F_1 Rijk Zwaan Zaadteelt, Zaadhandel B.V.) spinach (*Spinacea oleracea* L.) from April 3th to August

17th 2014 and repeated once from April 5th 2015 to August 18th 2015.

Spinach seeds were direct-sowed in 3 litres pots or were germinated and grown in 128, 200 and 288 (17.37, 13.90 and 6.18 cm^3 $cell^{-1}$ respectively) plastic plug trays filled with a 1:1 (v/v) mix of *Sphagnum maguellanicum* peat and river waste. When plug seedlings reached to the transplant stage, they were transplanted into 3 litres pots filled with the same growing media.

Seedlings were sprayed with BAP (6-benzyl amino purine) (SIGMA EC 214-927-5) (Sigma-Aldrich Co., St. Louis, MO, USA) solutions (0 and 100 mg L^{-1}) when the first true leaf pair were developed and the following 7, 14 or 21 days (pre-transplant). Additionally, seedlings without pre-transplant treatment were BAP-sprayed when they were transplanted and 7, 14 or 21 days after (post-transplant). BAP was previously diluted in ethanol 80%. Direct-seeded seedlings were BAP-sprayed as pre-transplant plug seedlings.

Plants were irrigated as needed with high quality tap water (pH: 6.64 and electrical conductivity of 0.486 dS m^{-1}) using intermittent overhead mist and one weekly fertigation (1N:0.5P:1K:0.5Ca v/v) (Stage 2: 50 mg L^{-1} N; Stage 3-4: 100 mg L^{-1} N; pot: 150 mg L^{-1} N) according to Styer and Koranski [23] was included. The volume per pot varied according to container volume.

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. The mean air temperatures ranged between 12.36 to 16.09°C and mean photosynthetic active radiation ranged between 12.46 to 17.33 mole m^{-2} day^{-1} during the experiments. The plants arrangement at a density of six plants m^{-2} avoided mutual shading.

Plants for destructive measurements were harvested (five per treatment) at emergence and at 20-day intervals during the 140 days of the experiments. 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 hours. The number of leaves was recorded and each leaf area was determined using the ImageJ® (Image Processing and Analysis in Java) software.

2.2 Assessed Variables

The rate of leaf appearance (RLA) was calculated as the slope of the number of fully expanded leaves versus time (in weeks). The relative growth rate (RGR) was calculated as the slope of the regression of the natural logarithm of the whole plant on a DW basis versus time (in days). The rate of leaf area expansion (RLAE) was calculated as the slope of the regression of the natural logarithm of total leaf area versus time (in days). The specific leaf area on a FW basis (SLA) was calculated as the ratio between the area of the new individual leaf and leaf FW. The mean net assimilation rate (NAR) was calculated as follows:

$$NAR = \frac{k_w W_0 e^{k_w t}}{A_0 e^{k_a t}}$$

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 (cm^2); k_a: RLAE ($days^{-1}$); t: time (in days) at the midpoint of the experimental period and e: base of natural logarithms.

The allometric coefficients between root and shoot were calculated as the slope of the straight-line regression of the natural logarithm of the root DW versus the natural logarithm of the shoot DW. On the other hand, the allometric coefficients between leaf blades + petiole and the stem were calculated as the slope of the straight-line regression of the natural logarithm of the leaf blade + petiole DW versus the natural logarithm of the stem DW.

Total soluble carbohydrate (TSC) concentration analysis was performed at the final sampling of the pot experiments (leaves) using the phenol-sulphuric method. A sample of 100 mg dried leaves was hydrolysed keeping it in boiling water bath for 3 hours with 5 ml of 2.5 H-HCl and cool root temperature. The hydrolysed solution was neutralised with solid sodium carbonate. The concentrated sulfuric acid breaks polysaccharides, which react with phenol to produce a yellow-gold colour. A standard glucose curve is performed and the absorption was measured with a Carl Zeiss DMR spectrophotometer at 490 nm.

2.3 Experimental Design and Statistical Analysis

The experimental design was a randomized factorial with three blocks of five single-pot

replications of each treatment combination (seeding routine × plug cell volume × BAP application time). Since there were no significant differences between the two yearly experiments, they were considered together (n = 6). Data are pooled by two consecutive years and were subjected to three-way analysis of variance (ANOVA). STATISTICA 8 (StatSoft) software was used for statistical analysis and the assumptions of ANOVA were checked. Least significant differences (LSD) values were calculated. Means were separated by Tukey's tests ($P \leq 0.05$). Slopes from straight-line regressions were tested using the SMATR package [24].

3. RESULTS

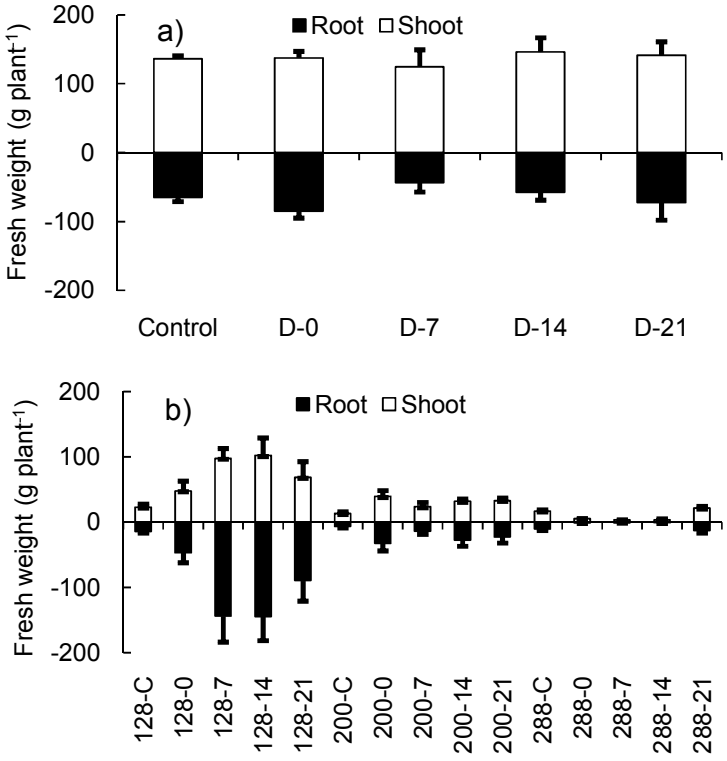
3.1 Fresh Biomass Accumulation

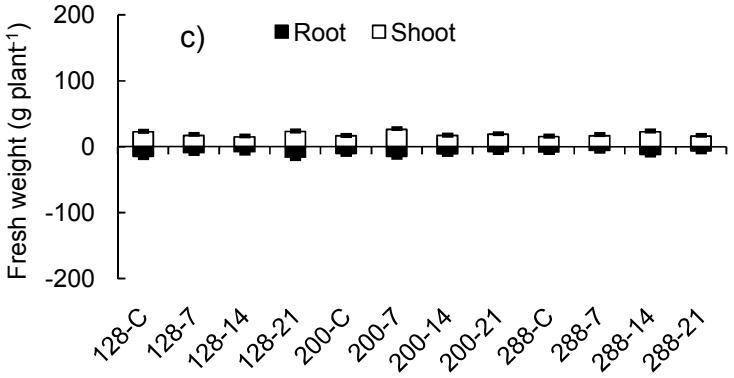
Direct-seeded plants showed the highest shoot FW without significant differences between control plants and BAP-sprayed ones (Fig. 1a) at the end of the experiment. On the other hand, the use of plug trays significantly decreased biomass accumulation in both roots and shoots for control plants, although seedlings from the higher plug cell volume showed the lower biomass accumulation decrease (Fig. 1b). In the same way, a BAP spray increased plant FW, although the effects have been linked to the pre-transplant BAP spray time and the plug cell volume. A post-transplant BAP spray did not change FW regarding control plants (Fig. 1c).

When shoot FW was check again root FW, a positive relationship ($r^2 = 0.889$) was found (Fig. 2).

3.2 Leaf Area Development

The higher both total leaf area and individual leaf area were found in plants direct-seeded and in those from the higher plug cell volume (128 plug cell tray^{-1}). A BAP spray increased total leaf area but decreased individual leaf area in direct-seeded plants; the effects enlarged according to the BAP application time increases. In transplanted seedlings, the response was linked to plug cell volume and BAP application time (Table 1). Control direct-seeded plants showed higher RLAE and RLA than BAP-sprayed ones. Although the BAP spray responses were linked to plug cell volume, RLAE and RLA increments were found (Table 1). In control direct-seeded plants, SLA was lower than transplanted ones. On the other hand, the higher plug cell volume, the lower SLA. A BAP spray on direct-seeded plants decreased SLA, with erratic results in the transplanted ones (Table 1).





ANOVA	Total FW
Sowing routine (A)	***
Plug cell volume (B)	***
BAP application time (C)	***
A x B	***
A x C	ns
B x C	ns
A x B x C	ns

*Significance ***.001 'ns' No significant*

Fig. 1. Shoot and root fresh weights from spinach plants direct-sowed (a) or from plants sowed and growth in plug cell trays with three different plug cell volume (128, 200 and 288 cell tray^{-1}). Seedlings were sprayed with a 100 mg L^{-1} BAP solutions when the first true leaf pair were developed (-0) and the following 7, 14 or 21 days (-7, -14 and -21 respectively) at the pre-transplant (b) or post-transplant (c) stages. The standard errors over each bar and the significance of interactions (ANOVA) have been indicated

Fig. 2. The relationship between shoot and root FW, included two initiation routines, three plug cell volume and eight BAP spray application time. Control plants have been indicated (full symbols). The straight-line regression was Shoot FW = 1.86 Root FW - 0.33 (r^2 = 0.889; P < 0.001)

3.3 Photo Assimilate Acquisition and Partitioning

Control direct-seeded plants showed the higher RGR and NAR values than plants grown in plug trays. The lower plug cell volume, the lower both RGR and NAR. The effect of a BAP spray on these growth parameters showed a significant increase in direct-seeded plants and an erratic response related to pre-transplant plug cell volume and application time in transplanted ones. An inverse LAR relationship was found as well. On the other hand, direct-seeded plants partitioned about the same photo-assimilate proportion between roots and shoots, while plug-grown plants partitioned a higher-photo assimilate proportion to shoots; a BAP spray on increased this response in the last (Table 2).

When all NAR and LAR data were plotted together, positive relationships between NAR and RGR (r^2 = 0.776) (Fig. 3a) and between NAR and SLA (r^2 = 0.633) (Fig. 3c) were found. On the contrary, a weak negative LAR versus RGR relationship (r^2 = - 0.282) (Fig. 3b) were found.

The higher glucose content were found in direct-seeded plants. A BAP spray significantly increased it over controls (Fig. 4a). In plug trays-grown plants the higher plug cell volume the higher glucose content. A pre-transplant BAP spray increased some glucose values (Fig. 4b). When BAP spray was applied at the post-transplant stage, only plants from the smaller plug cell volume (288-plug cell tray^{-1}) showed significant increments (Fig. 4c). Shoots showed the higher increases in glucose content than roots and leaves.

Table 1. Changes in both total and individual leaf area, the rate of leaf area expansion (RLAE) the rate of leaf appearance (RLA) and specific leaf area (SLA) in spinach plants direct-sowed or from plants growth in plug cell trays with three different plug cell volume and sprayed with a single pre- or post-transplant 100 mg L^{-1} BAP at different time. The significance of interactions (ANOVA) has been indicated. Treatments are as in Fig. 1. Data are pooled by two consecutive years

	Leaf area (cm^2 plant^{-1})	Leaf area (cm^2 leaf^{-1})	RLAE (cm^2 cm^{-2} day^{-1})	RLA (leaves week^{-1})	SLA (cm^2 g^{-1})
Direct-seeded					
C	1650.62	98.05	0.0469	0.1668	13.01
D-0	1805.96	92.63	0.0490	0.1989	9.50
D-7	2250.42	82.63	0.0425	0.1819	10.76
D-14	2664.26	65.41	0.0450	0.1797	12.04
D-21	2945.15	55.77	0.0457	0.1780	12.00
LSD	230.33	8.89	5.85	81.06	1.46
BAP Pre-transplant					
128-C	315.99	24.20	0.0264	0.0947	14.17
128-0	741.43	29.62	0.0286	0.1320	16.69
128-7	1428.49	52.50	0.0342	0.1494	14.79
128-14	1407.96	54.34	0.0311	0.1364	14.20
128-21	945.54	44.87	0.0303	0.1118	14.43
200-C	266.30	17.31	0.0256	0.0829	16.35
200-0	618.90	33.56	0.0244	0.0746	18.10
200-7	394.14	29.07	0.0261	0.0675	18.38
200-14	481.17	31.34	0.0258	0.0581	20.00
200-21	505.14	34.64	0.0259	0.0847	14.44
288-C	57.64	19.22	0.0212	0.0795	17.38
288-0	58.23	7.87	0.0203	0.1010	16.25
288-7	92.30	6.00	0.0199	0.0814	17.47
288-14	213.60	5.73	0.0180	0.0915	15.20
288-21	312.66	22.01	0.0256	0.0835	15.49
LSD	56.27	2.85	0.0026	0.067	1.27

	Leaf area (cm^2 $plant^{-1}$)	Leaf area (cm^2 $leaf^{-1}$)	RLAE (cm^2 cm^{-2} day^{-1})	RLA (leaves $week^{-1}$)	SLA (cm^2 g^{-1})
BAP Post-transplant					
128-C	316.13	24.50	0.0309	0.0836	14.19
128-7	281.73	19.84	0.0299	0.1007	16.94
128-14	241.96	19.42	0.0314	0.0961	16.37
128-21	347.80	21.29	0.0322	0.1092	15.29
200-C	266.19	20.19	0.0281	0.0871	16.11
200-7	373.60	30.23	0.0336	0.0908	14.51
200-14	258.48	24.19	0.0308	0.0745	15.24
200-21	298.24	18.63	0.0308	0.1012	15.78
288-C	243.02	19.88	0.0303	0.0869	16.25
288-7	270.60	24.56	0.0284	0.0749	16.30
288-14	301.48	26.00	0.0313	0.0799	13.73
288-21	270.47	21.94	0.0304	0.0869	16.68
LSD	29.17	2.56	0.0038	0.0098	1.65

ANOVA	Total leaf area	Individual leaf area	RLAE	RLA	SLA
Sowing routine (A)	***	***	***	***	***
Plug cell volume (B)	***	***	**	***	**
BAP application time (C)	***	***	**	***	*
A x B	***	***	**	***	**
A x C	***	***	**	***	*
B x C	***	***	*	***	*
A x B x C	ns	ns	ns	ns	ns

*Significance ***.001; **.01; *.05; 'ns' No significant*

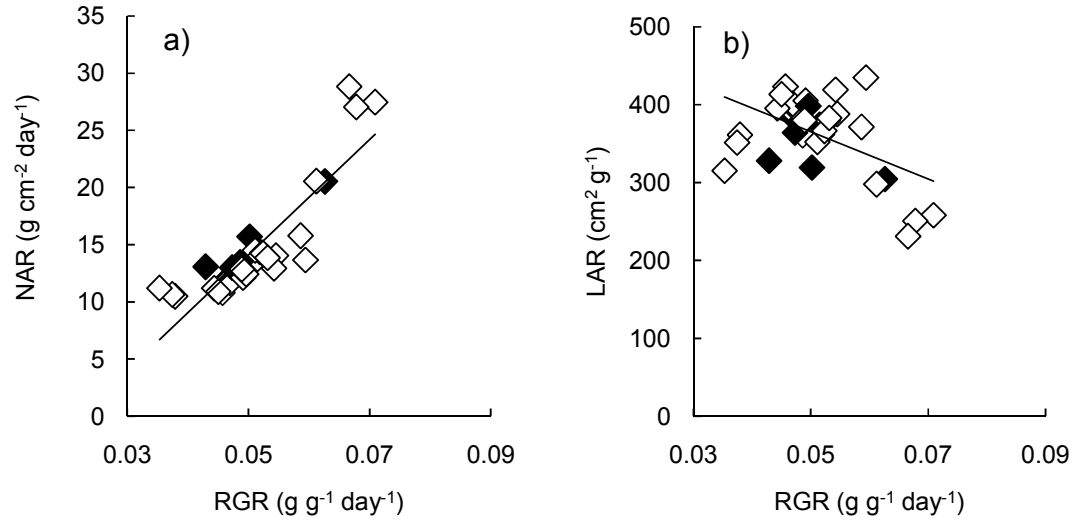
Table 2. Changes in RGR, NAR, LAR and allometric relationships between roots and shoots in spinach plants direct-sowed or from plants sowed and growth in plug cell trays with three different plug cell volume and sprayed with a single pre- or post-transplant 100 mg L^{-1} BAP. The significance of interactions (ANOVA) has been indicated. Treatments are as in Fig. 1. The probability of the slope being zero was P < 0.001 for RGR. Data are pooled by two consecutive years

	RGR (g g^{-1} day^{-1})	NAR (g cm^{-2} day^{-1}) (x 10^{-5})	LAR (cm^2 g^{-1})	Root: Shoot β
Direct-seeded				
C	0.0626	20.54	304.73	1.142
D-0	0.0709	27.45	258.32	1.099
D-7	0.0662	26.35	251.23	1.082
D-14	0.0678	27.03	250.84	1.114
D-21	0.0666	28.83	230.97	1.134
LSD	0.0082	1.46	25.29	0.412
BAP Pre-transplant				
128-C	0.0502	15.72	319.35	0.815
128-0	0.0515	13.77	374.02	0.794
128-7	0.0594	13.65	435.07	0.817
128-14	0.0586	15.78	371.37	0.832
128-21	0.0545	14.04	388.15	0.854
200-C	0.0464	11.94	388.54	0.829
200-0	0.0379	10.49	361.44	0.775
200-7	0.0374	10.64	351.53	0.788
200-14	0.0353	11.19	315.42	0.759
200-21	0.0462	11.67	395.84	0.752

	RGR ($g\ g^{-1}\ day^{-1}$)	NAR ($g\ cm^{-2}\ day^{-1}$) ($x\ 10^{-5}$)	LAR ($cm^2\ g^{-1}$)	Root: Shoot β
288-C	0.0429	13.07	328.29	0.916
288-0	0.0472	11.92	396.03	0.822
288-7	0.0457	10.80	423.27	0.804
288-14	0.0469	11.69	401.26	0.823
288-21	0.0443	11.20	395.48	0.807
LSD	0.0069	1.27	37.67	0.086
BAP				
Post-transplant				
128-C	0.0519	14.35	361.62	0.812
128-7	0.0486	13.47	360.76	0.714
128-14	0.0511	14.51	352.20	0.795
128-21	0.0529	13.93	379.75	0.752
200-C	0.0473	12.99	364.16	0.816
200-7	0.0542	12.93	419.22	0.816
200-14	0.0523	14.28	366.28	0.788
200-21	0.0491	12.12	405.13	0.784
288-C	0.0496	12.45	398.30	0.830
288-7	0.0450	10.89	413.27	0.788
288-14	0.0531	13.86	383.10	0.756
288-21	0.0489	12.87	379.82	0.755
LSD	0.0053	1.38	38.97	0.083

ANOVA	RGR	NAR	LAR	β
Sowing routine (A)	***	***	**	***
Plug cell volume (B)	***	**	*	*
BAP application time (C)	*	*	**	ns
A x B	***	**	*	**
A x C	*	**	**	*
B x C	*	*	*	ns
A x B x C	ns	ns	ns	ns

*Significance ***.001; **.01; *.05; 'ns' No significant*



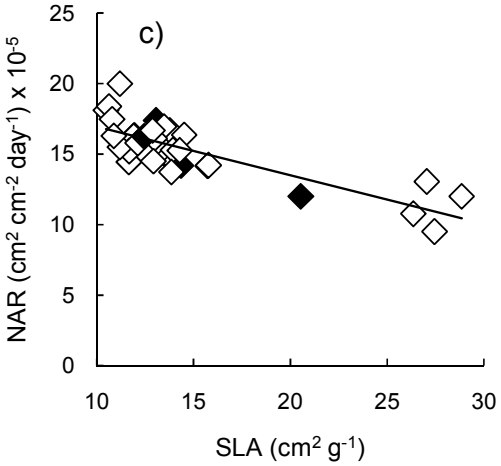
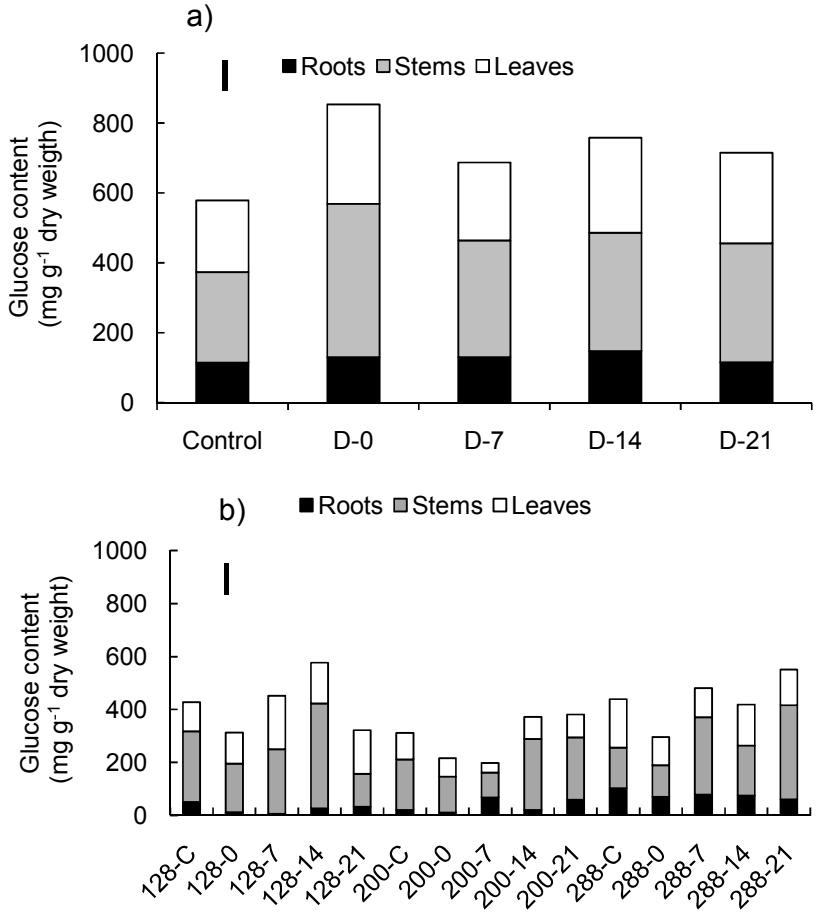
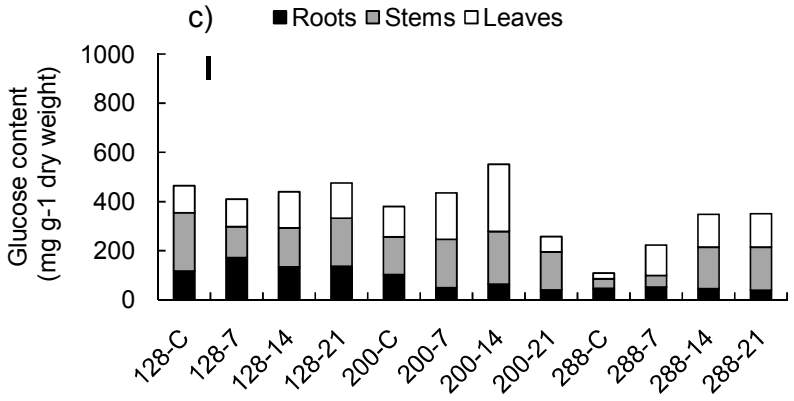


Fig. 3. Relationships between NAR (a), LAR (b) and RGR in spinach plants direct-sowed or from plants sowed and growth in plug cell trays with three different plug cell volume and sprayed with a single pre- or post-transplant 100 mg L^{-1} BAP at different times. Fig. 3c showed NAR and SLA relationship as well. Linear regression equation are NAR = 516.14 RGR – 11.65 (r^2 = 0.776; P < 0.001); LAR = -3184.90 RGR + 524.03 (r^2 = 0.282; P < 0.05); NAR = -0.35 SLA + 20.38 (r^2 = 0.633; P < 0.001). Control plants have been indicated (full symbols)





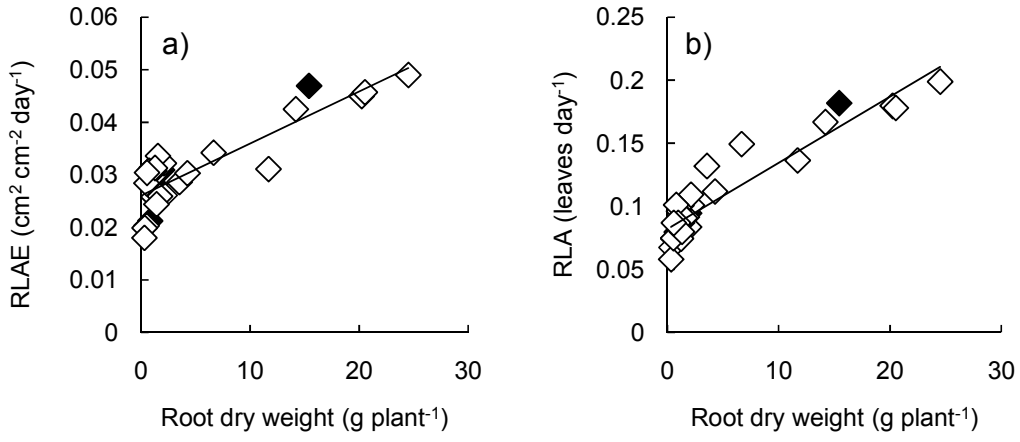
ANOVA	Total glucose content
Sowing routine (A)	***
Plug cell volume (B)	***
BAP application time (C)	*
A x B	***
A x C	*
B x C	*
A x B x C	ns

*Significance ***.001; **.01; *.05; 'ns' No significant*

Fig. 4. Glucose concentration in roots, leaves and shoots in spinach plants direct-sowed or from plants sowed and growth in plug cell trays with three different plug cell volume and sprayed with a single pre- or post-transplant 100 mg L^{-1} BAP at different times. Treatments are as in Fig. 1. Vertical lines indicate least significant differences (LSD) and the significance of interactions (ANOVA) has been indicated. Data are pooled by two consecutive years

3.4 Growth Rates and Root Dry Weight Relationships

Positive relationships between RLAE (r^2 = 0.753 P < .001) (Fig. 5a), RLA (r^2 = 0.879 P < .001) (Fig. 5b), RGR (r^2 = 0.756 P < .001) (Fig. 5c), NAR (r^2 = 0.904 P < .001) (Fig. 5d), glucose content (r^2 = 0.627 P < .001) (Fig. 5e) and root DW were found. On the other hand, negative relationship between SLA (r^2 = 0.632 P < .001) and root DW was found as well (Fig. 5f).



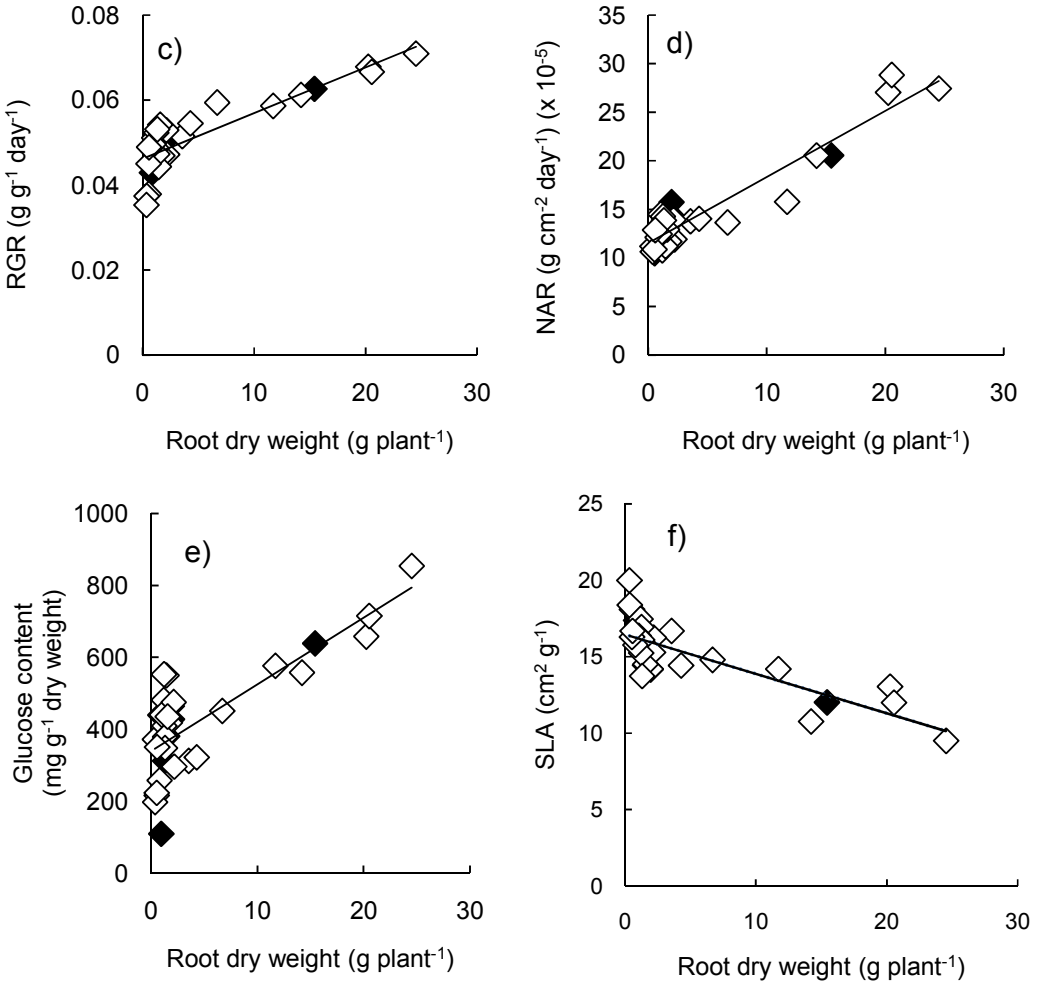


Fig. 5. Relationships between RLAE (a), RLA (b), RGR (c), NAR (d), glucose content (e), SLA (f) and root DW in spinach plants direct-sowed or from plants sowed and growth in plug cell trays with three different plug cell volume and sprayed with a single pre- or post-transplant 100 mg L^{-1} BAP at different times. The straight-line regressions were RLAE = 0.001 RDW + 0.026 (r^2 = 0.753; P < 0.001); RLA = 0.0052 RDW + 0.082 (r^2 = 0.879; P < 0.001); RGR = 0.0011 RDW + 0.046 (r^2 = 0.756, P < 0.001); NAR = 0.709 RDW + 11.52 (r^2 = 0.904; P < 0.001); Glucose content = 18.55 RDW + 3384.96 (r^2 = 0,627; P < 0.001); SLA = -0.258 RDW + 16.45 (r^2 = 0.632 P < 0.001). Control plants have been indicated (full symbols)

4. DISCUSSION

With the development of modern horticulture, the plug cell culture has become prevailing in many greenhouses during nursery. The plug tray technology is a powerful technique for saving agricultural resources and controlling environment of root systems, but a meta-analysis on the effects of pot size showed that root growth responds directly to impedance and decrease root growth and leaf area [25,26,27]. In this way, the significantly higher shoot FW in spinach direct-seeded plants from Fig. 1 would not be an unexpected result, although shoot FW increase in plug-grown vegetables exogenously BAP-sprayed has been previously indicated [1,2,19,28]. Plug cell volume during nursery must be understood as a spinach abiotic stress because it reduces yield below optimum levels.

Although spinach is a high yield plant, shoot FW accumulation in our experiments were lower than commercial yield and would be linked to pot management. Poorter et al. [27] have indicated

that pot size in scientific experiments seems to have received little consideration in the scientific literature and let explain differences between commercial spinach yield and our shoot FW results (Fig. 1a).

On the other hand, a trait did not usually keep in mind is root FW. For instance, the higher root FW was found in direct-seeded control spinach plants and in those limited by space but sprayed with BAP (Fig. 1a). On the other hand, Fig. 1b showed that shoot FW accumulation is positively related to root FW. Puig et al. [29] and Chen et al. [30] have concluded that plants can sense the volume of the rooting space available and respond accordingly. On the other hand, roots are a major source of cytokinins in plants, which are synthesized in roots and transported to shoots [31].

Plant development is regulated and coordinated by the activity of several hormones, which including cytokinins. They may act remote from their sites of synthesis to regulate responses to environmental stimuli [32]. The cytokinin function has been linked to different abiotic stresses [10,12] including plug cell volume during nursery [1,2,13,14,15,19]. Alteration of endogenous levels of cytokinins, in response to stress, indicates their involvement in abiotic stress. Although plant responses to cytokinins have been evaluated most via their external application, stressful conditions are also known to enhance their endogenous levels via uptake and biosynthesis [5]. As an example, abiotic stress such as salinity dramatically decreased the cytokinin content of the plant and especially tomato shoots [33]. Root restriction responses were found to be associated with the reduced supply of root-synthesized cytokinins [7,25].

In a previous report, Di Matteo et al. [2] described some of the physiological responses to a limited plug cell volume and the effect of different single BAP spray concentrations in spinach. However, the time when exogenous cytokinins are applied, it is important as well to consider both natural synthesis and synthetic exogenous applications, for which the composition and concentration of cytokinins in the site of action might be quite different from those in the site of application [20]. Differences in FW accumulation in spinach when the pre- or post-transplant BAP applications tested at different times are in agreement with this hypothesis and previous lettuce and celery results [16].

Spinach yield is mainly supported by leaf growth, for which total leaf area developed is the first step to optimizing yield. The shoot apical meristem (SAM) produces lateral organs at a regular interval (phyllochron) during the vegetative phase [34]. Leaf primordia are initiated at the flanks of a group of undifferentiated and proliferative cells within SAM, which requires the activity of the KNOX I genes for its establishment and maintenance [35,36]. Leaf growth process can be subdivided into two phases: the proliferative first phase is driven through the increase in cell mass by the synthesis of macromolecular cell constituents, coupled with cell division. In the second phase, growth continues by cell expansion [3]. In the SAM, several hormones, including auxin, cytokinin, and gibberellin, act in combination to regulate meristem function [37,38]. Cytokinin is generally considered to promote mitotic cell division in the shoot and positively regulates cell division [39]. Cytokinins mainly trigger physiological responses through the regulation of gene expression [40].

To characterize leaf area development and root restriction relationships, four growth parameters can be analyzed: First, the rate of leaf appearance (RLA) as an estimator of plastochron length, which involve leaf initiation rate. Second, the rate of leaf area expansion (RLAE), which quantify leaf growth. Third, the individual leaf area, which showed RLAE and expansion length relationships. Fourth, the capacity of leaf photo-assimilates acquisition associated with leaf thickness and estimated through the specific leaf area (SLA).

Our results showed that both RLA and RLAE were higher in direct-seeded plants and increased according to cell volume increase in transplanted plants (Table 1). It has been showed that plastochron may be altered in transgenic plants with reduced cytokinin levels [41], which explain the effect of a single BAP spray to override root restriction. In the same way, Shani et al. [42] showed the effect of cytokinin on leaf growth rate. Both a decrease in the plastochron length and an increase in leaf expansion are accompanied by the SAM size increase through the synthesis of high-molecular-weight substances essential for cell growth. Plant tissues and organs rich in cytokinins are known to attract the assimilate translocation and increased the sink capacity of the benzyl adenine-treated leaves [43].

A decrease in the plastochron and the increase in apex size needs the presence of non-limiting sugar supply [44]. Direct sensing through glucose sensors and indirect sensing via a variety of energy and metabolite sensors [45] perceive glucose signals. Cytokinins are a hormone with clear links to sucrose sensing and signaling. This feature has led to the suggestion that both play a role in integration of growth and development between shoots and roots [46]. According to this, the higher glucose contents in spinach direct-seeded plants and those BAP-sprayed were found (Fig. 4). In transplanted spinach plants, the higher plug cell volume, the higher glucose content, with a positive response to an early BAP spray. Poorter and Sack [47] indicated that sink organs can potentially stimulate sugar supply by activating their consumption rate, thereby increasing their sink strength. The relative carbon allocation to a particular organ must be regarded as a function of source and sink activities of all parts of the plant regulate by the relative photo-assimilates allocation [48]. Differences in plant allometries shown in Table 2 are in agreement with these assumptions.

The final size of plant organs, such as leaves, is controlled mainly by environmental and genetic factors that must spatially and temporally coordinate cell expansion and cell cycle activity [49]. Total leaf area was mainly controlled by individual leaf area and RLA. Hepworth and Lenhard [50] indicated that the final size of plant organs must be regulated in response to the developmental stage and the environment to optimally exploit the plant's surroundings. Direct-seeded plants showed the higher individual leaf area, while the higher plug cell volume, the higher single leaf area, but a meta-analysis of our results showed a negative relationship between RLA and individual leaf area with significant differences in BAP-sprayed plants related to BAP application time (Table 1).

The amount of light absorbed by a leaf and the diffusion pathway of CO_2 through its tissues depend, at least partially, on its thickness [51]. The potential leaf photosynthetic capacity, expressed on a leaf area basis, is determined by the intrinsic cell physiological capacity and by the structural components; leaf thickness affects the variation in CO_2 transfer conductance [52]. On the other hand, Poorter et al. [53] suggest that in response to variation in resources availability, the major part of plant adjustments in leaf area is driven by plastic changes in specific leaf area (SLA). Data from Table 1 showed that SLA is particularly sensitive to changes in the external environment and the internal functioning of the plant. The negative relationship between the net assimilation rate (a growth parameter closely related to photosynthetic rate) and SLA (Fig. 3c) are in agreement with Oguchi et al. [54], who showed a strong correlation between leaf thickness and the light-saturated rate of photosynthesis per unit leaf area.

Variation in the relative growth rate (RGR) has traditionally been linked to three key traits: the leaf net carbon assimilation rate (NAR), leaf area ratio (LAR) and SLA [55]. Although leaf area determined plant capacity of light interception, RGR, which ultimate quantify biomass accumulation, is greatly influenced by photosynthetic efficiency [56]. On the other hand, Poorter et al. [27] suggested that the differences in RGR of plants growing in different pot sizes are always smaller than the differences in biomass at the end of the experiment. This implies that the physiological and morphological factors that underlie the variation in biomass will also be affected to a smaller extent than biomass itself. Direct-seeded spinach plants showed the higher both RGR and NAR values with a significant increase in BAP-sprayed ones. On the other hand, the lower plug cell volume, the lower RGR and NAR in transplanted spinach plants (Table 2). Shipley [57] indicated that, in general, NAR was the best general predictor of variation in RGR, in agreement with our results from Fig. 3. On the other hand, Shi et al. [25] showed that root restriction often depresses photosynthetic capacity but indicated that the mechanism for this reduction remains unclear. However, cytokinin is known to stimulate the expression of photosynthetic enzymes like Rubisco [58]. Plants synthesize different cytokinin-ribosides but not all have biological activity [59], although the higher root system, the higher the zeatin ribosides [60]. In any case, we can show positive relationships between RLAE (Fig. 5a), RLA (Fig. 5b), RGR (Fig. 5c), NAR (Fig. 5d), and glucose content (Fig. 5e) and root DW. In the same way, we found a negative SLA-root DW relationship as well (Fig. 5f).

For optimal development of the plant as a whole, root and shoot biomass have to be balanced. A plausible control mechanism for organ growth is the regulation of relative assimilate allocation [48]. Although there is broadly coordinated interspecific variation between biomass allocations aboveground vs belowground organs,

these relationships can be largely modified by plant phenotypic adjustments to variable environmental conditions [52]. Sink strength and source activity can be altered by endogenous hormones and environmental factors. Auxin and cytokinin have major roles in source nutrient remobilization and sink development [61]. Direct-seeded spinach plants showed a balanced allocation in roots and shoots while the lower plug cell volume, the lower photo-assimilate partitioning to spinach shoots. On the other hand, a single BAP spray change photo-assimilate partitioning to favor shoots (Table 1). These results suggest that the root restriction change photo-assimilate allocation and BAP (an alleviator to abiotic stress) increased shoots as the main plant sink.

5. CONCLUSIONS

Plug cell volume can be considered as an abiotic stress, which decreases spinach yield. From a grower's point of view, the use of direct-seeded plants or large plug cell volume would increase spinach growth. A promising approach to increasing crop productivity is the use of the exogenous cytokinins (BAP), but a commercial suggestion needs for future calibration.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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