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Could Sodium Benzoate Enhance Broad Bean Salinity Tolerance?

III. Antimicrobial Potency, Antioxidant Activity and Phytochemical Constituents

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Abstract: The objective of the present study was to investigate the impact of seed presoaking in sodium benzoate (SB) on seawater (SW)- stressed broad bean (*Vicia faba* L.) at early germination stage. Methanolic extracts of 7- day old seedlings were prepared to assess their antimicrobial efficacy, antioxidant criteria and phytochemical profile. The results obtained indicated that the extracts of all treatments has no effect on *Streptococcus pyogenes* or *Proteus vulgaris*. Meanwhile, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Candida albicans* were greatly inhibited by the extracts especially those of SB- treated and/ or SW- stressed seedlings. It was recorded also that SB could enhance the ability of some extracts to scavenge diphenyl-picrylhydrazyl (DPPH) and H_2O_2 with accompanied increase in their reducing power and total antioxidant activity. Through phytochemical analysis, proteins, alkaloids, saponins and calcones were found to be absent from the extracts of all the considered treatments. However, reducing sugars, carbohydrates, amino acids, phenols, flavonoids, tannins, phloba-tannins, steroids, terpenoids and anthraquinones were all present but to

various levels. Based on the resulted data, seed presoaking in SB was recommended to enhance the antimicrobial and antioxidant activity of SW- stressed broad bean seedlings.

Keywords: broad bean; sodium benzoate; seawater; antimicrobial; antioxidant; phytochemicals

INTRODUCTION

Sodium benzoate (SB) is one of the most commonly used preservatives employed to inhibit microbial deterioration of various foodstuffs because of its bactericidal and bacteriostatic action without considerable toxicity or taste[1]. In food industry, SB is usually involved as highly efficient antimicrobial agent to conserve food products typically those with an acidic pH such as sweets, soft drinks, fresh juices, canned foods, sauerkrauts, beverages and margarines[2]. In such a way, food spoilage by the action of microbes can be suppressed or at least slowed down preventing the loss of food quality, edibility or nutritional value and thus allowing its longer storage.

Beside its antimicrobial potency, the antioxidant activity of SB is well documented especially against hydroxyl radical[3-4]. In this connection, Mangge *et al*.[5] considered SB as one of the powerful antioxidative food additives that could scavenge noxious radicals generated during food storage. It was suggested also by Sarkar *et al*.[6] that SB added to some fruit drinks during their cold storage might not only inhibit microbial growth, but also scavenge free radicals that could be formed due to oxidation over time.

In an aqueous medium, SB is converted into benzoic acid which is the active form of the preservative[7]. Generally, SB is preferred over benzoic acid because of the more solubility of SB in water[8]. The United States Food and Drug Administration has paid great attention to SB and found that it is safe to human body when consumed in the allowable dozes[7]. According to the European Commission, SB usage in food products is limited to 0.5% or below[9]. In other words, the acceptable daily intake levels of SB as recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) is 0-5 mg for each kg body weight[10].

In a series of recent studies undertaken by Mickky[11-12], SB was proven to be an effective presoaking agent that could enhance broad bean (*Vicia faba* L.) salinity tolerance at early germination phase. In these studies, broad bean was chosen to be considered depending on its fundamental nutritive value as a legume for both human and animals. In addition, the possible use of seawater (SW) in irrigation sector as an alternative to the diminishing freshwater was addressed in such a proposed protocol of using SB in seed presoaking to ameliorate the ill impact of salinity on seed germination and consequent seedling growth. Therefore, the current study was conducted to evaluate the antimicrobial activity of SW- stressed faba bean seedlings after seed pre-treatment with SB. In addition, the antioxidant properties of the considered seedlings were assessed. Since the antimicrobial and antioxidant activities of any plant are often ascribed to its phytochemical constituents, the present work also involved intensive phytochemical screening of the extracts of the seedlings under the studied conditions.

EXPERIMENTAL

Plant Material and Experimental Design: Some uniform seeds of broad bean (*Vicia faba* L., cultivar Giza 3, pedigree G.1*NA29) were superficially disinfected with 0.01 M $HgCl_2$ then thoroughly washed with distilled water to rid of the adhered disinfectant. Afterward, the seeds were soaked in distilled water,

0.25 mM SB or 0.50 mM SB each for 8 hours. The seeds were then germinated in dark at 25±2°C for 7 days during which each of the three sets was sprayed with distilled water, 10% SW or 25% SW so as to obtain 9 treatments.

Preparation of the Plant Extracts: Seedlings from each of the 9 groups were firstly oven- dried at 50°C for 5 days. The dry tissues were then grinded in an electrical blender to obtain fine powder from which 20 g was taken to be soaked in methanol for 72 hours with vigorous shaking at 180 rpm. The extracts were then filtered and raised up to 200 ml with methanol.

Estimation of Antimicrobial Potency: Filter paper disc procedure was followed as cited from Murray *et al*.[13] to screen the antimicrobial potentiality of the considered extracts against 11 pathogenic bacteria and only one fungus. Stock cultures of the tested microbes (*Erwinia carotovora*, *Enterobacter cloacae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Shigella flexneri*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Candida albicans*) were obtained from the Microbiology Laboratory, Faculty of Medicine, Mansoura University, Egypt. For bacteria, nutrient agar medium was used whereas Czapek dox agar medium was employed for the fungus. For comparison, methanol was implicated as negative control while ampicillin and clotrimazole were assayed as antibacterial and antifungal agents; respectively. The microbial susceptibility was evaluated by recording the diameter of inhibition zone in mm 24-48 hours post-inoculation.

Estimation of Antioxidant Activity: To assess 2, 2'-diphenyl-1-picrylhydrazyl (DPPH)- scavenging activity of the studied faba bean extracts, the method of Ebrahimzadeh *et al*.[14] was adopted. In addition, IC_{50} could be computed graphically as the mass of the plant tissue in mg needed to scavenge 50% of the radical. Also, the antiradical power of the extracts (mathematical reciprocal of IC_{50}) was calculated. Moreover, H_2O_2- scavenging potentiality of the extracts was determined as described by Ruch *et al*.[15]. The extracts were also assessed for their reducing power and total antioxidant activity following the spectrophotometric procedures of Dorman and Hiltunen[16] and Prieto *et al*.[17]; respectively.

Estimation of Phytochemical Constituents: Following the protocols of Harborne[18] and Kokate[19], the assayed plant extracts were subjected to phytochemical screening to identify their profile of reducing sugars, carbohydrates, amino acids, proteins, phenols, alkaloids, flavonoids, saponins, tannins, phloba-tannins, steroids, terpenoids, anthraquinones and calcones. When a phytochemical category was identified to be absent from the plant extract, - sign was denoted while +, ++ and +++ were given to represent the presence of certain category in low, medium and high amount; respectively.

Statistical Analysis: The performed analyses were carried out three times for each treatment and only the mean values were represented ± the standard deviation. For both of the antimicrobial and antioxidant tests, the pooled data were analyzed using "CoHort/ CoStat" software version 6.311 in an ANOVA (Analysis Of Variance) style to obtain the values of LSD (Least Significant Difference) at $p \leq 0.05$. Small letters were then given referring to statistically different values. Moreover, the degree of significant variation among all the treatment (significance degree) was described as * (low), ** (medium), *** (high) or ns (non-significant).

RESULTS AND DISCUSSION

Modifications in Antimicrobial Potency: The impact of SW stress on the antimicrobial efficiency of broad bean presoaked in SB is represented in the present research in **table 1**. The pooled data manifested that the methanolic extracts of SW- stressed and unstressed seedlings in presence or absence of SB could not affect the growth of *Streptococcus pyogenes* or *Proteus vulgaris* at all so these bacterial genera were not included in the representative table. The most powerful inhibitory action of the extracts was recorded when dealing with *Bacillus subtilis* and *Candida albicans* both followed by *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. For the extracts of unsalted seedlings, seed presoaking in 0.25 or 0.50 mM SB increased their inhibiting effect on *Bacillus subtilis* but such increase was statistically non-significant at $p \leq 0.05$. Similarly, seed presoaking in 0.25 mM SB enhanced the inhibitory effect of the extracts of seedlings sprayed with 10% SW but also to statistically non-significant level. For *Candida albicans*, seed presoaking in 0.50 mM SB could significantly enhance the retarding action of SW- unstressed seedlings extracts. Also, 0.25 mM SB had the same influence on 25% SW-stressed seedlings extracts. SB at 0.25 mM could also significantly stimulate the inhibiting action of 0 and 10% SW- treated seedlings on *Pseudomonas aeruginosa*. For *Staphylococcus* whether *aureus* or *epidermidis*, application of SB and/ or SW reduced the inhibitory action of the extracts against these two bacterial species.

Table 1: Effect of sodium benzoate (SB) on the antimicrobial activity of broad bean seedlings irrigated with seawater (SW). Data listed are the mean values of three replica ± standard deviation with least significant difference (LSD) at 0.05.

Parameter / Treatment	Clear Zone Diameter (mm)				
	Erwinia carotovora	***Enterobacter cloacae***	***Pseudomonas aeruginosa***	***Klebsiella pneumoniae***	***Escherichia coli***
0% SW + 0 mM SB	$8.3^{b} \pm 1.15$	$0^{b} \pm 0$	$0^{d} \pm 0$	$0^{b} \pm 0$	$0^{b} \pm 0$
0% SW + 0.25 mM SB	$8^{b} \pm 0$	$0^{b} \pm 0$	$7^{b} \pm 0$	$0^{b} \pm 0$	$0^{b} \pm 0$
0% SW + 0.50 mM SB	$9^{a} \pm 0$	$6.97^{a} \pm 0.15$	$0^{d} \pm 0$	$0^{b} \pm 0$	$0^{b} \pm 0$
10% SW + 0 mM SB	$0^{c} \pm 0$	$0^{b} \pm 0$	$6^{c} \pm 0$	$0^{b} \pm 0$	$0^{b} \pm 0$
10% SW + 0.25 mM SB	$0^{c} \pm 0$	$0^{b} \pm 0$	$8^{a} \pm 0$	$8.03^{a} \pm 0.15$	$9.3^{a} \pm 0$
10% SW + 0.50 mM SB	$0^{c} \pm 0$	$0^{b} \pm 0$	$0^{d} \pm 0$	$0^{b} \pm 0$	$0^{b} \pm 0$
25% SW + 0 mM SB	$0^{c} \pm 0$	$0^{b} \pm 0$	$0^{d} \pm 0$	$0^{b} \pm 0$	$0^{b} \pm 0$
25% SW + 0.25 mM SB	$0^{c} \pm 0$	$0^{b} \pm 0$	$0^{d} \pm 0$	$0^{b} \pm 0$	$0^{b} \pm 0$
25% SW + 0.50 mM SB	$0^{c} \pm 0$	$0^{b} \pm 0$	$0^{d} \pm 0$	$0^{b} \pm 0$	$0^{b} \pm 0$
LSD at $p \leq 0.05$	0.66	0.09	$1.36e^{-7}$	0.09	$1.52e^{-7}$
Significance Degree	***	***	***	***	***

Continued …

Parameter / Treatment	Clear Zone Diameter (mm)				
	Shigella flexneri	***Bacillus subtilis***	***Staphylococcus aureus***	***Staphylococcus epidermidis***	***Candida albicans***
0% SW + 0 mM SB	$0^{b} \pm 0$	$9.67^{b} \pm 0.58$	$6.83^{a} \pm 0.29$	$6.93^{b} \pm 0.12$	$0^{b} \pm 0$
0% SW + 0.25 mM SB	$0^{b} \pm 0$	$11.33^{ab} \pm 1.15$	$0^{b} \pm 0$	$0^{c} \pm 0$	$0^{b} \pm 0$
0% SW + 0.50 mM SB	$7^{a} \pm 0$	$10.33^{ab} \pm 1.53$	$0^{b} \pm 0$	$0^{c} \pm 0$	$7.6^{a} \pm 1.22$
10% SW + 0 mM SB	$0^{b} \pm 0$	$12.33^{a} \pm 3.06$	$6.93^{a} \pm 0.12$	$11.33^{a} \pm 2.08$	$8.33^{a} \pm 0.58$
10% SW + 0.25 mM SB	$0^{b} \pm 0$	$12.67^{a} \pm 2.52$	$0^{b} \pm 0$	$7.5^{b} \pm 0.5$	$0^{b} \pm 0$
10% SW + 0.50 mM SB	$0^{b} \pm 0$	$0^{d} \pm 0$	$0^{b} \pm 0$	$7^{b} \pm 0$	$0^{b} \pm 0$
25% SW + 0 mM SB	$0^{b} \pm 0$	$7^{c} \pm 0$	$0^{b} \pm 0$	$0^{c} \pm 0$	$0^{b} \pm 0$
25% SW + 0.25 mM SB	$0^{b} \pm 0$	$0^{d} \pm 0$	$0^{b} \pm 0$	$0^{c} \pm 0$	$8^{a} \pm 1$
25% SW + 0.50 mM SB	$0^{b} \pm 0$	$6.67^{c} \pm 0.58$	$0^{b} \pm 0$	$0^{c} \pm 0$	$0^{b} \pm 0$
LSD at $p \leq 0.05$	$4.82e^{-8}$	2.56	0.18	1.23	0.96
Significance Degree	***	***	***	***	***

For *Erwinia carotovora*, *Enterobacter cloacae* and *Shigella flexneri*, spraying the seedlings with 10 or 25 % SW caused the extracts to exhibit no action on these bacteria; and the same was recorded for *Klebsiella pneumoniae* and *Escherichia coli* but by 25% SW only. With respect to the first 3 bacterial genera (*Erwinia carotovora*, *Enterobacter cloacae* and *Shigella flexneri*), 0.50 mM SB significantly enhanced the retarding impact of the extracts of SW- unstressed seedlings. Regarding the last 2 genera (*Klebsiella pneumoniae* and *Escherichia coli*), it is the 0.25 mM concentration of SB that enhanced the extracts action against them.

The antimicrobial activity of SB was similarly recorded *via* various *in vitro* trials. In this context, SB could inhibit the growth of *Bacillus subtilis*, *Bacillus mycoides*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus flavus*, *Fusarium oxysporum*, *Candida albicans*, *Trichoderma harsianum* and *Penicillium italicum*[1]. As a proposed mechanism of action, benzoates were found to interfere with the utilization of acetate needed for the function of energy- rich compounds resulting in blockage of cell metabolism[20]. In addition, Everis[21] ascribed SB inhibitory action to its deleterious impact on the genetic material of the microbial cell and/ or its cellular membrane.

Also, Adeshina and Onaolapo[2] working on *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Micrococcus sp*. recorded inhibition zones caused by SB ranging from 13.5 to 36.5 mm with an overall notice that SB exerted more effective action against the tested Gram positive bacteria than the Gram negative ones. Such trend was apparent in the current study where the extracts exerted no inhibiting action on the Gram negative *Proteus vulgaris*. Gram negative bacteria are known to be relatively resistant to the action of many antimicrobial agents because of their outer phospholipids membrane which is characterized by structural lipopolysaccharide components that make their cell wall impermeable to antimicrobial agents[22].

The enhanced antimicrobial activity of the extracts in the present study due to seedling treatment with SW (more notably at 10%) in presence or absence of SB may be attributed to the stress- induced accumulation

of certain metabolites with antimicrobial potency (e.g., phenols, flavonoids, phloba-tannins and steroids). This assumption is clear from the results represented herein in **table 3**.

Modifications in Antioxidant Activity: Results in **table 2** showed the antioxidant activities of the alcoholic extracts of the considered seedlings in the form of DPPH- and H_2O_2- scavenging activity, reducing power and total antioxidant activity. It was clear from these results that 0.25 and 0.50 mM SB markedly increased the ability of the extracts of SW- unstressed seedlings to scavenge DPPH radical and so did 0.25 mM SB in case of the extracts of 25% SW- stressed seedlings ($p \leq 0.05$). In consequence, IC_{50} of the aforementioned extracts decreased causing their antiradical power to increase. SB, more apparently at 0.50 mM, increased H_2O_2- scavenging potential as well as the total antioxidant activity (but to lesser extent) of almost all the seedlings extracts. Regarding their reducing power, both of the checked concentrations of SB could increase the reducing power of the extracts of 0 and 25% SW- stressed seedlings.

As assumed for the antimicrobial activity, increased antioxidant activity of the extracts in the present study under the treatment with SB and/ or SW might be caused by the increased amount of certain phytochemicals in the extracts such as phenols, flavonoids and steroids. In consistence with the results recorded herein, the total antioxidant activity of guava juice to which SB was added as a preservative during 180 storage days was significantly higher than that of the corresponding juice without SB[23].

Table 2: Effect of sodium benzoate (SB) on the antioxidant activity of broad bean seedlings irrigated with seawater (SW). Data listed are the mean values of three replica ± standard deviation with least significant difference (LSD) at 0.05.

Parameter / Treatment	Antioxidant Activity		
	DPPH- scavenging activity (%)	**IC_{50} (mg ml^{-1})**	**Antiradical power (ml mg^{-1})**
0% SW + 0 mM SB	66.22^{e} ± 2.82	48.65^{d} ± 2.10	0.021^{c} ± 0.001
0% SW + 0.25 mM SB	93.81^{a} ± 0.77	10.99^{g} ± 2.52	0.095^{a} ± 0.025
0% SW + 0.50 mM SB	90.59^{b} ± 0.70	30.36^{e} ± 0.28	0.033^{b} ± 0
10% SW + 0 mM SB	92.20^{ab} ± 0.27	27.34^{f} ± 2.59	0.037^{b} ± 0.004
10% SW + 0.25 mM SB	84.49^{c} ± 1.24	50.88^{cd} ± 0.69	0.020^{c} ± 0.001
10% SW + 0.50 mM SB	76.38^{d} ± 2.85	52.37^{c} ± 0.22	0.019^{c} ± 0
25% SW + 0 mM SB	75.22^{d} ± 1.38	66.59^{b} ± 1.23	0.015^{c} ± 0
25% SW + 0.25 mM SB	82.58^{c} ± 1.21	51.54^{c} ± 1.18	0.019^{c} ± 0.001
25% SW + 0.50 mM SB	75.53^{d} ± 0.63	69.24^{a} ± 1.15	0.015^{c} ± 0.001
LSD at $p \leq 0.05$	2.72	2.28	0.012
Significance Degree	***	***	***

Continued …

Parameter / Treatment	Antioxidant Activity		
	H_2O_2- scavenging activity (%)	Reducing power (A)	Total antioxidant activity (A)
0% SW + 0 mM SB	$12.96^{b} \pm 2.06$	$0.60^{d} \pm 0.04$	$2.07^{c} \pm 0.25$
0% SW + 0.25 mM SB	$9.44^{cde} \pm 1.92$	$0.88^{a} \pm 0.05$	$1.69^{d} \pm 0$
0% SW + 0.50 mM SB	$17.69^{a} \pm 0.72$	$0.78^{ab} \pm 0.01$	$2.16^{c} \pm 0.19$
10% SW + 0 mM SB	$6.28^{ef} \pm 0.36$	$0.72^{bc} \pm 0.15$	$2.40^{b} \pm 0.15$
10% SW + 0.25 mM SB	$3.08^{f} \pm 0.13$	$0.59^{d} \pm 0.04$	$2.20^{bc} \pm 0.13$
10% SW + 0.50 mM SB	$7.14^{de} \pm 0.16$	$0.63^{cd} \pm 0.03$	$2.19^{bc} \pm 0.11$
25% SW + 0 mM SB	$3.85^{f} \pm 1.47$	$0.69^{bcd} \pm 0.08$	$2.06^{c} \pm 0.10$
25% SW + 0.25 mM SB	$10.79^{bc} \pm 1.02$	$0.77^{ab} \pm 0.04$	$2.79^{a} \pm 0.07$
25% SW + 0.50 mM SB	$9.86^{bcd} \pm 4.49$	$0.74^{bc} \pm 0.07$	$2.80^{a} \pm 0.09$
LSD at $p \leq 0.05$	3.23	0.12	0.24
Significance Degree	***	***	***

Modifications in Phytochemical Constituents: Phytochemical analysis of broad bean methanolic extracts represented herein in **table 3** revealed that the extracts of all the considered treatments lacked the presence of proteins, alkaloids, saponins and calcones as well. On the other hand, the other chemical categories searched for in the present study were all present in the extracts of all treatments but to various levels as indicated by the intensity of positive colors or precipitates except for tannins that seemed to be present to almost similar levels in all the extracts. For reducing sugars however, the extracts of bean seedlings treated with 25% SW appeared to contain more sugars than the extracts of unsalinized seedlings or those salinized with 10% SW especially when the seeds were soaked in 0.25 mM SB rather than 0.50 mM SB. When searching for carbohydrates, it was recorded that seed pre-treatment with 0.50 mM SB had a stimulating effect on carbohydrates amount in SW- sprayed or unsprayed seedlings.

Also, SW at 10% or 25% increased the amount of amino acids in absence or presence of SB whose 50 mM concentration seemed to be more efficient than 25 mM. Meanwhile, SW at 10% only raised phenols amount in the extracts of bean seedlings with SB presoaking; and SW at 25% only raised flavonoids amount in the extracts of the seedlings without SB presoaking. On contrary, SW at the two tested doses decreased the level of terpenoids in the extracts of SB- treated or untreated bean. Regarding steroids, 10% as well as 25% SW upgraded terpenoids level when compared with the extracts of their unstressed relatives. Also, 0.50 mM SB could increase the amount of such class in 10% SW- stressed seedlings. With respect to phloba-tannins, seed presoaking in the higher concentration of SB raised their amount in the extracts of 10% SW- treated seedlings.

The upgrade in the amount of some phytochemicals recorded herein as a result of SW treatment may be an adaptive response of the seedlings to cope with salt stress since many secondary metabolites are well established to help the plants withstand stressful conditions. On the other hand, exposing some plants to stress may have negative impact on the synthesis of certain metabolites.

Table 3: Effect of sodium benzoate (SB) on the phytochemical constituents of broad bean seedlings irrigated with seawater (SW). (+: Low concentration, ++: Medium concentration, +++: High concentration, -: Absent).

Parameter / Treatment	Phytochemical Constituents						
	Reducing sugars	Carbo--hydrates	Amino acids	Proteins	Phenols	Alkaloids	Flavonoids
0% SW + 0 mM SB	+	+	+	-	+	-	+
0% SW + 0.25 mM SB	+	+	+	-	+	-	+
0% SW + 0.50 mM SB	+	++	++	-	++	-	++
10% SW + 0 mM SB	+	+	++	-	+	-	++
10% SW + 0.25 mM SB	+	+	++	-	+++	-	+
10% SW + 0.50 mM SB	+	+++	++	-	+++	-	+
25% SW + 0 mM SB	++	+	+	-	+	-	+++
25% SW + 0.25 mM SB	+++	+++	++	-	+	-	+++
25% SW + 0.50 mM SB	++	+++	+++	-	+	-	+++

Continued …

Parameter / Treatment	Phytochemical Constituents						
	Saponins	Tannins	Phloba-tannins	Steroids	Terpenoids	Anthra-quinones	Calcones
0% SW + 0 mM SB	-	+	++	+	+++	-	-
0% SW + 0.25 mM SB	-	+	+	+++	+++	-	-
0% SW + 0.50 mM SB	-	+	+	+	++	-	-
10% SW + 0 mM SB	-	+	+	+++	+	-	-
10% SW + 0.25 mM SB	-	+	+	+	+	-	-
10% SW + 0.50 mM SB	-	+	+++	+++	+	-	-
25% SW + 0 mM SB	-	+	+	++	+	-	-
25% SW + 0.25 mM SB	-	+	+	+	+	-	-
25% SW + 0.50 mM SB	-	+	+	+	++	-	-

CONCLUSION

Based on the findings obtained herein, it is recommended to presoak broad bean seeds in SB especially when treating bean with SW during early germination and juvenile stages to enhance its antimicrobial and/ or antioxidant activity.

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