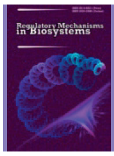

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Impact of photoperiod and soybean genotypes (E-genes) on the composition of root exudates, growth and biofilm formation of rhizosphere microbiota of soybean isogenic lines

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The study is dedicated to analyzing the impact of photoperiod on the quantitative and qualitative composition of root exudates in *Glycine max* (L.) Merr. with varying photoperiod sensitivity, as well as their influence on the growth and biofilm formation of the soil bacteria *Bradyrhizobium japonicum* and *Bacillus subtilis*. Soybean near-isogenic lines (NILs) differing in the allelic state of photoperiod sensitivity genes (*E1–E3*) were used. The plants were grown for 14 days in a controlled environment chamber under two light conditions: a short day (9 hours) and a long day (16 hours). Root exudates were collected for biochemical analysis of carbohydrate, protein, amino acid, phenol, and flavonoid content, and to evaluate their effects on bacterial growth and biofilm formation. The results demonstrate that the photoperiod significantly affects the composition of root exudates, with the effect depending on the allelic state of the E genes in the plant genotype. Under short-day conditions, short-day lines (Clark and L80-5879) and the day-neutral line L63-3117 exhibited an increase in monosaccharide content. In root exudates of all lines, short days led to a decrease in soluble protein content, and in lines with dominant *E1–E3* genes, a reduction in amino acid content was observed. An exception was the day-neutral line L71-920 (*e1e2e3*), in which amino acid content increased under short-day conditions. Short days significantly increased phenol content in the exudates of L63-3117 but decreased it in L71-920. In lines with the dominant *E3* gene (Clark and L63-3117), short days led to an increase in flavonoid content, while in other lines it decreased. Root exudates of the cv. Clark under short-day conditions inhibited the growth of *B. japonicum*, likely due to reduced amino acid and protein content, which serve as nitrogen sources, and an increase in flavonoids, which may exert an inhibitory effect. Exudates from line L80-5879 did not affect rhizobial growth, while those from L63-3117 inhibited it, likely due to low amino acid content. In contrast, exudates of L71-920 under short days stimulated rhizobial growth, which correlated with increased amino acid content, potentially acting as chemoattractants. Biofilm formation by *Br. japonicum* was suppressed by exudates of short-day lines regardless of photoperiod, whereas in day-neutral lines (L63-3117 and L71-920), short-day conditions significantly stimulated biofilm formation. For *B. subtilis*, exudates of all lines under long-day conditions inhibited biofilm formation, but under short days, lines with dominant *E1–E3* genes promoted it, likely due to increased monosaccharide content as a carbon source. In co-cultivation of rhizobia and bacilli, exudates from most lines under short days enhanced biofilm formation, except in L71-920, where a decrease in monosaccharide content was observed. These results highlight the importance of genotypic photoperiod sensitivity in forming the composition of root exudates and their influence on the rhizosphere microbiota in response to photoperiod. This has important implications for understanding the mechanisms regulating symbiotic interactions between soybean and microorganisms, and for optimizing agronomic practices for soybean cultivation across different latitudes.

Keywords: *Glycine max*; rhizobacteria; photoperiod sensitivity; biofilm, root secretion; flavonoids; phenolic compounds; amino acids; carbohydrate.

Introduction

Photoperiod is an important factor of plant development, influencing metabolic and physiological processes. Phenotypically, photoperiod affects the timing of flowering, extending or shortening the vegetative phase. Thus, the perception and response to photoperiod are crucial for maximizing plant productivity and adapting to seasonal changes (Roeber et al., 2022; Gendron et al., 2023). Photoperiod sensitivity is genetically determined. In soybean (*Glycine max* (L.) Merr.), a system of early flowering genes (*E*-genes: *E1–E9*, *J*, *Dt1*, and *Dt2*) has been identified (Cao et al., 2016; Lin et al., 2021). The structure, regulation, and function of most of these genes have been studied. Notably, the *E1*, *E2*, and *E3* genes repress flowering under long-day conditions, but their influence extends beyond ontogenetic development (Li et al., 2021). Physiological and metabolic changes induced by photoperiod, along with sensitivity to it, determine plant interactions with environmental factors, including plant-bacteria interactions.

Soil microbiota, including plant growth-promoting rhizobacteria (PGPR), significantly influence soybean growth. PGPR include a wide range of soil bacteria, such as *Bacillus mucilaginosus*, *B. edaphicus*, *B. circulans*, *Burkholderia* sp. (Pettigrew, 2008), *Acidithiobacillus ferrooxidans*, *Enterobacter hormaeche* (Meena et al., 2016; Raghavendra et al., 2016), *Arthrobacter* spp., *Paenibacillus glucanolyticus* (Jaiswal et al., 2016), *Pseudomonas fluorescens*, *Rhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Allorhizobium*, *Mesorhizobium*, *Frankia*, *Azospirillum*, *Azotobacter*, *Acetobacter*, Clostridia (Hayat et al., 2012), and others. Symbiotic relationships with Rhizobiales, which fix atmospheric nitrogen, are specific to soybeans (Gage, 2004). The species composition and diversity of the rhizosphere microbiome depend on physical and chemical factors, including soil quality, moisture, and pH, all of which also affect microbial function (Upadhyay et al., 2022). The secretion of root exudates by plants, which significantly modulates the species composition of the rhizosphere microbiota, is an integral component of the plant–soil feedback loop. This process is driven by the plant's needs and involves the release of exudates that create conditions conducive to bacterial colonization of the rhizosphere (Vives-Peris et al., 2020). Rhizosphere colonization is an important step in the development of symbiotic relationships. However, optimal colonization conditions do not necessarily guarantee the establishment of close symbioses, due to the diverse soil microbial community and competitive interactions. Biofilm formation is a key bacterial trait that facilitates successful colonization of the rhizosphere and rhizoplane in competitive environments (Danhorn &

Fuqua, 2007). Root exudate secretion depends not only on plant needs but also on biotic and abiotic factors. In return, plants obtain easier access to soil minerals essential for development. Such interactions are essential for plant productivity, as plants allocate 5–21% of photoassimilates (Upadhyay et al., 2022), while other studies report 11–40% of fixed carbon being directed toward exudate secretion (Zhalnina et al., 2018; Du et al., 2021). That is, plants invest a substantial portion of their accumulated resources in secreting compounds into the soil to maintain the surrounding environment and create optimal conditions around the root for more effective bacterial colonization. Moreover, root exudates play a crucial role in establishing species-specific interactions between members of the order Rhizobiales and the family Fabaceae, particularly between *Bradyrhizobium japonicum* and *Glycine max* (Gage, 2004). These exudates include low-molecular-weight compounds such as sugars, amino acids, phenolics, and organic acids, as well as high-molecular-weight compounds including polysaccharides, polyuronic acids, and proteins (Badri & Vivanco, 2009; Cesari et al., 2019). The intensity, quantity, and quality of exudates depend on the phenological stage, environmental conditions, nutrient availability, stress factors, and interactions with fungi, bacteria, insects, and other plants (Vives-Peris et al., 2020). Photoperiod may also influence exudate composition. Studies have demonstrated differences in assimilate accumulation and transport in plants exposed to varying photoperiods (Chatterton & Silvius, 1979, 1980). As a result, assimilate delivery to roots may differ, which in turn affects the intensity and composition of root exudates that serve as chemoattractants for bacteria. The effect of photoperiod on the quantitative and qualitative composition of root exudates remains poorly studied. However, some studies have demonstrated that photoperiod can influence the rate of exudation. For example, Pramanik et al. (2000) showed that a reduction in day length decreases the exudation rate of certain organic acids, such as benzoic and 4-hydroxybenzoic acids, in *Cucumis sativus*. However, the study did not take into account the plant's photoperiod sensitivity, which is determined by its genotype. Watt & Evans (1999) reported that this process is regulated by circadian rhythms, with exudation rates increasing during light periods. Guo et al. (2023), in their study on *Acorus calamus* under wetland conditions, demonstrated that long photoperiod exposure enhances bacterial nitrification and denitrification activity in the rhizosphere. These effects were attributed to increased photosynthetic activity under long-day conditions, which leads to a higher content of photosynthates in root exudates, and stimulates microbial metabolic processes as well as plant–microorganism interactions. In previous research we showed that a 9-hour photoperiod increased the abundance of nitrate-reducing bacteria and decreased ammonifying isolates in the soybean rhizosphere (Hlushach & Zhmurko, 2021).

Thus, the aim of the study was to determine the role of photoperiod and soybean genotype (*E* genes) in modulating the quantitative and qualitative composition of root exudates, and to assess the effect of these secretions on the growth and biofilm formation of *Bradyrhizobium japonicum* and *Bacillus subtilis*, both individually and in cocultivation.

Materials and methods

Near-isogenic lines (NILs) of soybean (*Glycine max* (L.) Merr.) differing in photoperiod sensitivity genes (*E*-series) were used, derived in the cv. Clark genetic background. NILs share a common genotype but differ in one or more loci, making them ideal for studying *E*-gene control of physiological and biochemical processes in response to photoperiod. For the experiment, lines with known allelic states of the *E1–E5* and *E7* genes were selected (Tasma & Shoemaker, 2003), with *E1*, *E2*, and *E3* genes in varying allelic states: short-day lines – cv. Clark (*e1E2E3E4e5E7*) and L80-5879 (*E1e2e3E4e5E7*); neutral-day lines – L63-3117 (*e1e2E3E4e5E7*) and L71-920 (*e1e2e3E4e5E7*). Seed germination energy was consistent across all isolines at 99%.

To assess the impact of root exudates on growth and biofilm formation, *Bradyrhizobium japonicum* (Kirchner) serogroup 634b, provided by the Institute of Agricultural Microbiology and Agroindustrial Production, NAAS, Chernihiv, Ukraine, and *Bacillus subtilis*, from the collection of the Department of Plant and Microorganisms Physiology and Biochemistry, Faculty of Biology, V. N. Karazin Kharkiv National University, were used. *Bradyrhizobium japonicum* 634b is a soybean-specific diazotroph (Krutylo & Nadkernychna, 2021), while *Bacillus subtilis* is a common representative of soil microbiota and a well-established model organism for biofilm research (Qin et al., 2022). Since diazotrophs are not the only constituents of soil microbial communities, using both strains allowed for the evaluation of soybean root exudate effects on biofilm formation both separately and in co-culture. *Bradyrhizobium japonicum* 634b was incubated for 3 days at 28 °C using mannitol-yeast medium with soil extract (SEB; Soil Extract Broth, g/L: yeast extract – 1 (LLC Hlr, Spain); mannitol – 10 (LLC Hlr, Ukraine); soil extract – 200 mL; distilled water – 800 mL; pH 7.0) (Atlas, 2005). *Bacillus subtilis* was grown for 24 hours at 28 °C with constant shaking (100 rpm) in commercial meat-peptone broth (MPB).

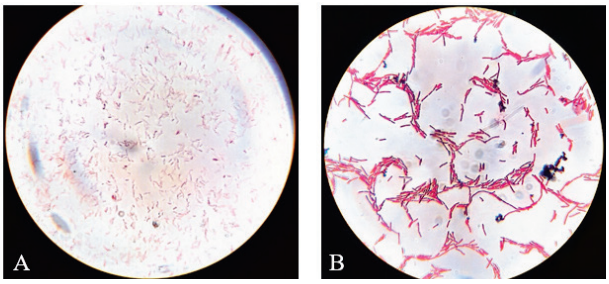

Fig. 1. Light micrographs (Zeiss Axiostar, Germany) of fuchsin-stained bacterial strains that were used in study (magnification, ×1000): *A – Bradyrhizobium japonicum* (Kirchner) 634b; *B – Bacillus subtilis*

Seeds of each isoline were sterilized with a 10% hydrogen peroxide solution (Ukraine) for 30 minutes and then germinated in sterile Petri dishes for 3 days. Subsequently, the seedlings were cultivated under hydroponic conditions in growth containers filled with 500 mL of sterile tap water. A single container held 15 plants of one isoline and was maintained at a temperature of 23–25 °C during the day and 18–19 °C at night, under either a short photoperiod (9 hours, experimental condition) or a long photoperiod (16 hours, control condition), until the formation of the first trifoliate leaf. The liquid containing root exudates was brought back to the initial volume of 500 mL and used for biochemical analyses and for evaluating the effects of root exudates on rhizobial growth and biofilm formation.

Carbohydrate content was determined using a micro-method based on the reduction of potassium ferricyanide ($K_3[Fe(CN)_6]$) to potassium ferrocyanide ($K_4[Fe(CN)_6]$) by the free hemiacetal hydroxyl group (Porro et al., 1981). Protein content was measured with Coomassie Brilliant Blue using the Bradford method (Bradford, 1976). Free amino acids were quantified using the standard ninhydrin method (Yemm & Cocking, 1955). Total phenols and flavonoids were determined after evaporating the root exudate solution to dryness at 50 °C. The residue was extracted with 96% ethanol for 1 hour at 25 °C. Phenols were measured using the Folin–Ciocalteu reagent, and flavonoids were quantified by a standard method with 5% $NaNO_2$ (LLC Hlr, Chine) and 10% $AlCl_3$ (LLC Hlr, Chine) (Fattahi et al., 2014). Results were expressed as milligrams of carbohydrates or micrograms of other compounds per plant, based on the total volume of the cultivation solution.

Effect of root exudates on the growth of B. japonicum 634b. For the blank, 5 mL of SEB medium and 2 mL of sterile tap water were added to a sterile test tube. In the test tubes, 2 mL of root exudates from isogenic lines of soybean grown under short (9 h) or long (16 h) photoperiods were added instead of water. A suspension of *B. japonicum* 634b was added to both the control and test tubes to achieve a final concentration of 2.7×10^7 cells/mL. All tubes were incubated for 3 days at 28 °C. On the third day, optical density was measured at 600 nm, and bacterial cell numbers were determined using a calibration curve based on the Vinogradsky-Shulgina-Brid method.

The influence of root exudates on bacterial biofilm formation was determined using a modified standard method in 24-well (6×4) plates with a well volume of 2 mL (Stiefel et al., 2016). Overnight liquid cultures of *B. japonicum* 634b and *B. subtilis* were prepared in cultivation media at 26 °C with constant shaking (100 rpm). Plates were thoroughly sterilized by soaking in concentrated bleach solution (20–30% sodium hypochlorite) for two days, followed by three washes with boiled, sterile distilled water. After washing, plates were dried in a laminar flow cabinet under a bactericidal lamp for 60 minutes before use. For blank wells, 0.5 mL each of SEB and MPB media (1 mL total), along with 1 mL of root exudates (filtered through a 0.22 µm Millex-GS bacterial filter), were added. Test wells contained 0.35 mL each of SEB and MPB media (0.75 mL total), 0.25 mL of bacterial suspension (10^8 CFU), and 1 mL of sterile root exudates. Each plate contained exudates from only one isogenic soybean line. Plates were incubated at 32 °C for 24 hours to allow biofilm formation. Biofilm formation was evaluated using acridine orange (AO; Sigma-Aldrich, USA), a fluorescent dye that binds to cellular DNA. The working AO solution was prepared by diluting 1 mL of 2% aqueous dye in 99 mL of Walpole's buffer (pH 4.0). After incubation, bacterial suspensions were aseptically removed, and each well was washed three times with 2 mL of 0.9% NaCl to remove non-adherent cells. Biofilms were fixed by drying the plates at 60 °C for 40 minutes (Kwasny & Opperman, 2010). After fixation, 2 mL of AO dye was added to each well and incubated in the dark for 15 minutes. The dye was then removed, and wells were washed three times with 2 mL of 0.9% NaCl. To elute the dye, 2 mL of 0.9% NaCl was added; plates were covered with foil and shaken at 250 rpm for 10 minutes. Fluorescence intensity was measured using a BioTek FL600 plate fluorimeter (USA) with an excitation filter at 485 nm and an emission filter at 530 nm, maintaining consistent instrument sensitivity. Biofilm formation was quantified as the relative cell count, calculated as the ratio of the test fluorescence unit to the blank. A relative cell count below 2 arbitrary units (acceptance level) indicated no biofilm formation.

Data were analyzed using Statistica 10 (StatSoft Inc., 2011, USA). Mean values and standard deviations (SD) were calculated. Differences between groups were evaluated via one-way analysis of variance (ANOVA), with differences considered significant at P < 0.05 (Bonferroni correction was accounted).

Results

Under the influence of a short-day photoperiod, a trend of increasing the quantity of monosaccharides was observed in all soybean lines that possess at least one dominant *E1–E3* gene in their genotype. Among the total carbohydrate content in root exudates, the larger fraction consists of oligosaccharides, as shown in Table 1. A significant increase in the oligosaccharide content under short-day conditions was observed in the short-day cultivar Clark and the day-neutral line L71-920. However, this increase did not lead to a substantial rise in the overall carbohydrate content in the root exudates of the L71-920 line under short-day conditions, distinguishing this line from others. A significant decrease in both oligosaccharides and the total sugar content under short-day conditions was observed in the L80-5879 line. At the same time, the increase in the quantity of monosaccharides in the root exudates of the L63-3117 line under short-day conditions did not result in an increase in the total amount of soluble carbohydrates, as the rise in oligosaccharide content was not significant. Thus, a trend has been identified for day-neutral lines, which indicates that under short-day conditions, they do not increase the total content of soluble carbohydrates in root exudates compared to the natural long-day photoperiod.

Under the influence of a short-day photoperiod, a significant decrease in the content of soluble proteins in the root exudates of all soybean lines was observed, regardless of genotype (Table 2). At the same time, short-day treatment resulted in a reduction in the free amino acid content in the root exudates of lines whose genotype contained at least one dominant E1–E3 allele. Only the L71-920 line, which carries recessive e1–e3 alleles, demonstrated an increase in amino acid content under short-day conditions. Thus, an inverse correlation was observed between the contents of monosaccharides and amino acids.

Table 1

Effect of photoperiod on the content of soluble carbohydrates in root exudates of isogenic soybean lines for *E*-genes (mg/plant, n = 10, x ± SD)

Isoline	Parameter	Long-day	Short-day	Content change, %
cv. Clark	Monosaccharides	42.5 ± 2.7	71.5 ± 5.5***	+68.2
	Oligosaccharides	96.2 ± 7.8	215.1 ± 18.3***	+123.6
	Total	138.7 ± 9.3	286.5 ± 24.8***	+106.6
L80-5879	Monosaccharides	36.5 ± 2.8	51.3 ± 3.4**	+40.5
	Oligosaccharides	492.7 ± 43.5	173.6 ± 19.5***	−64.8
	Total	529.2 ± 48.8	224.9 ± 15.9**	−57.5
L63-3117	Monosaccharides	47.3 ± 3.9	63.7 ± 4.9*	+35.7
	Oligosaccharides	211.1 ± 21.5	226.3 ± 15.2	+7.2
	Total	258.3 ± 14.1	289.9 ± 16.8	+12.2
L71-920	Monosaccharides	117.5 ± 11.1	65.8 ± 7.1**	−44.0
	Oligosaccharides	130.4 ± 10.1	224.1 ± 25.1**	+71.9
	Total	247.9 ± 17.8	289.8 ± 29.1	+16.9

Notes: comparisons were made within each isogenic soybean line between the variant "short-day" and "long-day" for each of parameters; significant differences between the average values in the "short day" and "long day" columns for each isogenic line are marked with symbols based on one-way ANOVA with Bonferroni correction: * for P < 0.05, ** for P < 0.01, and *** for P < 0.001; the direction of the trend change in the amount of each parameter for each isoline under the influence of a short-day photoperiod is indicated in the "Content change" column.

Table 2

Effect of photoperiod on the content of soluble proteins and amino acids in root exudates of soybean NILs (µg/plant, n = 10, x ± SD)

Isolines	Soluble proteins			Amino acids		
	long-day	short-day	content change, %	long-day	short-day	content change, %
cv. Clark	92.8 ± 8.0	61.0 ± 4.3**	−34.3	14.2 ± 1.1	6.8 ± 0.9**	−52.1
L80-5879	43.5 ± 3.4	15.9 ± 1.4***	−63.5	18.3 ± 2.0	11.8 ± 1.0*	−35.5
L63-3117	57.2 ± 4.7	43.8 ± 5.2*	−23.4	11.3 ± 0.9	7.7 ± 0.8**	−31.9
L71-920	68.4 ± 3.1	44.2 ± 4.3**	−35.4	26.1 ± 2.3	39.3 ± 3.3**	+50.6

Note: see Table 1.

The effect of a short-day photoperiod results in significant changes in the quantity of phenolic compounds only in the root exudates of neutral-day soybean lines, with these changes being line-specific (Table 3). Specifically, the L63-3117 line shows an increase in phenol content under short-day conditions, whereas the L71-920 line exhibits a decrease. The influence of a short-day photoperiod on the flavonoid content in root exudates is particularly noteworthy. Lines that have the dominant *E3* gene in their genotype (cultivar Clark, L63-3117) significantly increase flavonoid levels under short-day conditions, while the other lines, in contrast, show a decrease.

Influence of root exudates on the growth of B. japonicum 634b. The effect of root exudates of the cv. Clark (*E2E3*) grown under short-day conditions leads to a significant decrease in the growth of *B. japonicum* 634b, compared with the effect of exudates under long-day conditions (Fig. 3). In contrast, under short-day conditions, the exudates of the L80-5879 line (*E1*) did not significantly affect rhizobial growth. A similar absence of effect was noted for the L63-3117 line (*E3*). An increase in the number of *B. japonicum* 634b cells under short-day conditions was observed only in the presence of root exudates from the L71-920 line (*e1e2e3*) in the cultivation medium.

Table 3
Effect of photoperiod on phenolic compounds and flavonoids content in root exudates of soybean isogenic lines (µg/plant, n = 10, Mean ± SD)

Isolines	Total phenols			Flavonoids		
	long-day	short-day	content change, %	long-day	short-day	content change, %
cv. Clark	5.63 ± 0.46	6.27 ± 0.44	+12.5	5.01 ± 0.17	6.35 ± 0.55*	+28.0
L80-5879	4.81 ± 0.43	3.98 ± 0.39	−16.7	4.46 ± 0.20	1.65 ± 0.14***	−62.2
L63-3117	3.17 ± 0.27	4.79 ± 0.22**	+50.0	0.39 ± 0.02	3.51 ± 0.26***	+775.0
L71-920	6.82 ± 0.76	2.90 ± 0.22*	−57.4	7.81 ± 0.48	0.14 ± 0.01***	−98.7

Note: see Table 1.

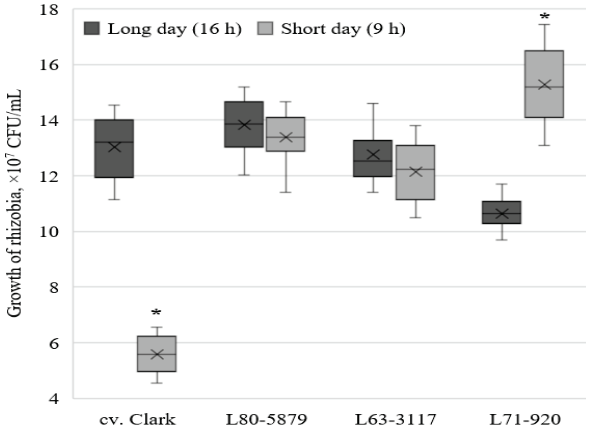

Fig. 2. Growth of *B. japonicum* 634b under the influence of root exudates of soybean NILs, grown under different photoperiod conditions ($\times 10^7$ CFU/mL; n = 10, x ± SD): comparisons were made within each isoline between the "short day" and "long day" variants; statistically significant variants are marked with "*" based on one-way ANOVA (P < 0.05) with Bonferroni correction; additionally, bacteria were grown without root exudates, with water added, and their growth was 9.8×10^7 CFU/mL

According to the results obtained (Table 4), it was established that in the absence of plant root exudates, the selected bacteria are capable of forming biofilms, as the relative number of bacterial cells in both monoculture and co-culture exceeds the acceptance threshold (2 arbitrary units). At the same time, an inhibitory effect of root exudates from short-day soybean lines on the formation of *B. japonicum* 634b biofilms was observed, regardless of the photoperiod under which the plants were grown. For neutral-day soybean lines, another trend was noted: under natural long-day conditions, their exudetes suppressed rhizobial biofilm formation, whereas under short-day conditions, they significantly stimulated biofilm development. The response observed for the neutral-day line L71-920 corresponds to the trend previously identified in the experiment evaluating the effect of its root exudates on rhizobial growth in liquid medium.

A somewhat different effect was observed in the formation of *B. subtilis* biofilms. Under long-day conditions, root exudates from all soybean lines inhibited biofilm formation. In contrast, under short-day conditions, the exudates of lines that had at least one dominant *E1-E3* gene promoted biofilm formation. However, it should be noted that in the Clark cultivar and L80-5879, the number of cells in the biofilm was significantly lower (under short-day) than in the variant without exudate application. Nevertheless, under short-day conditions (compared to long-day), the root exudates of plants that had at least one dominant *E1-E3* gene enhanced biofilm formation during co-cultivation of rhizobia and bacilli. A similar trend was observed during the cultivation of bacilli alone. In the case of co-cultivation, we can speak of an enhancement of biofilm formation, as the number of cells in the biofilm either increased significantly (Clark, *E2E3*) or remained at the level of the variant without exudates (L80-5879, *E1*, and L63-3117, *E3*). The root exudates of the L71-920 line (*e1e2e3*) grown under short-day conditions had an inhibitory effect on biofilm formation by rhizobia in co-culture with bacilli. A positive effect of root exudates on biofilm formation by bacilli together with rhizobia was observed for all lines under natural long-day conditions, except for L80-5879 (*E1*). This stimulatory effect was not observed when the bacteria were cultured separately.

Table 4
Biofilm formation by soil bacteria exposed to root exudates of NILs of soybean grown under different photoperiod (arbitrary units, n = 10, x ± SD)

Isolines	Bacteria	Long day	Short day	Trend change, %
cv. Clark	*Br. japonicum* 634b	1.34 ± 0.08 $^{(nf)}$	0.90 ± 0.13** $^{(nf)}$	−32.8
	Bacillus subtilis	1.32 ± 0.17 $^{(nf)}$	2.62 ± 0.13***	+98.5
	Br. japonicum 634b + *B. subtilis*	4.58 ± 0.32	10.36 ± 1.35**	+126.2
L80-5879	*Br. japonicum* 634b	1.29 ± 0.08 $^{(nf)}$	1.66 ± 0.10** $^{(nf)}$	+28.7
	Bacillus subtilis	1.78 ± 0.27 $^{(nf)}$	2.25 ± 0.11*	+26.4
	Br. japonicum 634b + *B. subtilis*	1.18 ± 0.11 $^{(nf)}$	4.86 ± 0.73**	+311.9
L63-3117	*Br. japonicum* 634b	1.15 ± 0.14 $^{(nf)}$	4.89 ± 0.49***	+325.2
	Bacillus subtilis	1.80 ± 0.13 $^{(nf)}$	5.29 ± 0.63**	+193.9
	Br. japonicum 634b + *B. subtilis*	2.48 ± 0.22	4.56 ± 0.36**	+83.9
L71-920	*Br. japonicum* 634b	0.92 ± 0.07 $^{(nf)}$	6.52 ± 0.75***	+608.7
	Bacillus subtilis	1.21 ± 0.16 $^{(nf)}$	1.65 ± 0.20* $^{(nf)}$	+36.4
	Br. japonicum 634b + *B. subtilis*	3.07 ± 0.37	1.67 ± 0.18** $^{(nf)}$	−45.6

Notes: the selected bacteria are biofilm-forming, as formation was observed without exudates (a.u.): *B. japonicum* 634b – 3.93 ± 0.53, *B. subtilis* – 5.89 ± 0.63, during co-cultivation – 5.52 ± 0.83; the letters "nf" indicate variants where biofilm formation was not detected (according to the method); comparisons were made within each isogenic soybean line between the "short-day" and "long-day" conditions for each parameter; significant differences between the average values in the "short day" and "long day" columns are marked with asterisks based on one-way ANOVA with Bonferroni correction: * for P < 0.05, ** for P < 0.01, and *** for P < 0.001; the trend percentage change of each parameter for each isoline under the influence of a short-day photoperiod is indicated in the "trend change" column.

Discussion

Role of photoperiod and soybean genotype in regulating bacteria growth and biofilm formation via root exudates. Plant growth and development regulation is significantly influenced by interactions with PGPR bacteria (Shah et al., 2021), whose species composition depends on many physical and chemical parameters of the rhizosphere – such as moisture, pH, soil quality, and the secretion of root exudates by plants (Canarini et al., 2019). Root exudates may contain compounds capable of regulating rhizosphere microbiome diversity. For example, organic acids and carbohydrates can serve as sources of carbon and energy, while amino acids and proteins can serve as nitrogen sources. Phenols (Chamam et al., 2013; Canarini et al., 2019) and flavonoids (Coronado et al., 1995; White et al., 2017) can regulate bacterial species composition. Root exudates play a significant role in establishing interactions with members of the order Rhizobiales, which form specific interactions with members of the Fabaceae family, including soybean. At the symbiosis initiation stage, flavonoids produced by the macrosymbiont stimulate the expression of rhizobial genes (nod) responsible for further interaction development (Gage, 2004).

Application of root exudates from the short-day cv. Clark grown under short-day conditions significantly reduced the growth of *B. japonicum* 634b. The increase in soluble carbohydrates (mono- and oligosaccharides) observed under short-day conditions was not a growth-promoting factor. Likely, protein and free amino acids (nitrogen sources), whose levels decreased in Clark's exudates under short-day conditions, play a decisive role in bacterial growth. Meanwhile, flavonoids, which increased under short-day conditions, may exert a repressive effect on rhizobial growth when Clark's root exudates are added to the cultivation medium. Flavonoids and other phenolic compounds may play dual roles, either stimulating or inhibiting bacterial growth, motility, and biofilm formation (Wang et al., 2022). Among all lines, Clark had the highest concentrations of soluble carbohydrates and flavonoids in its root exudates under both short- and long-day conditions. However, when its exudates were applied, biofilm formation by rhizobia was absent under both photoperiods. Notably, under short-day conditions (unlike long-day), biofilm formation by *Bacillus subtilis*, a typical soil bacterium, was observed. Likely, soluble carbohydrates as carbon sources serve as a significant trophic factor in *Bacillus* biofilm formation. It is interesting, that under long-day conditions, rhizobia and bacilli do not form biofilms separately, but a biofilm forms during co-cultivation. This suggests that interactions not only with the plant but also between bacteria are important. The enhanced biofilm formation under short-day conditions upon Clark exudate application confirms the importance of trophic factors in this process. Biofilm formation by bacteria is integral to their survival in the soil since it is a crucial factor in colonizing the environment, including the rhizoplane or rhizosphere. Successful colonization of these zones by rhizobia and/or bacilli is a basic for initiating interactions with plants (Rinaudi & Giordano, 2010). For gram-negative bacteria, biofilm formation is critical for survival in highly variable or adverse conditions, as they lack structures (such as spores) to endure such stress (Qin et al., 2022). Thus, by regulating bacterial biofilm formation, plants can influence rhizosphere and rhizoplane colonization by certain beneficial bacterial species. Moreover, colonization of the rhizoplane, especially by rhizobia, is essential for the development of more specific interactions with the plant. The genotype of the Clark cultivar have dominant *E2E3* genes, which are repressors of flowering under long-day conditions and thus prolong the vegetative growth phase (Lin et al., 2022). Consequently, under long-day conditions, the short-day Clark cultivar, due to extended vegetative growth, remains in environmental interaction for a longer ontogenetic period, including with microorganisms. In contrast, under short-day conditions, short-day plants accelerate development and have limited resources for establishing plant-bacteria interactions. Therefore, the suppression of rhizobial growth under short-day-derived Clark exudates is logical. Possibly, the development of specific interactions between rhizobia and soybean does not require a biofilm formation stage, which could explain its absence upon long-day Clark exudate application.

The absence of an effect of short-day-derived exudates from the short-day line L80-5879 (*E1*) on *B. japonicum* 634b growth indicates genotype-specific influence. While monosaccharide content in exudetes increases under short-day conditions, the total content of carbohydrates, soluble proteins, and free amino acids decreases. Additionally, flavonoid content in the exudates also decreases under short-day conditions. These trends result in the lack of growth stimulation. Rhizobial biofilm formation is inhibited, likely due to reduced free amino acid content (a nitrogen source) caused by photoperiod effects. However, the decrease in flavonoids and increase in monosaccharides promotes *B. subtilis* biofilm formation both separately and during co-cultivation with rhizobia. Thus, under short-day conditions, L80-5879 root exudates neither stimulate rhizobial growth nor biofilm formation. As in the Clark case, biofilm formation might not be essential for interaction establishment. Meanwhile, *Bacillus* biofilm formation, separately or in co-culture with rhizobia, depends on trophic factors (monosaccharides) and inter-bacterial interactions.

In the root exudates of the neutral-day line L63-3117 under short-day conditions, an increase in monosaccharides and phenolics (including flavonoids) and a decrease in amino acids were observed. These changes limited *B. japonicum* 634b growth in the culture medium. However, exudate application stimulated biofilm formation by both bacilli and rhizobia when cultured separately. Thus, despite a neutral-day response, the short-day photoperiod specifically affects L63-3117 metabolism, enhancing bacterial interactions. This may relate to the presence of the dominant *E3* allele in L63-3117's genotype. It is known that the *E3* allele encodes a functional phytochrome A3 protein (GmPhyA3), a photoreceptor sensitive to the ratio of red to far-red light. Kirakosyan et al. (2006) found that the quantity of isoflavones in the roots of the studied soybean lines varies depending on the presence of light or its spectral composition. The authors suggest that phytochromes interact with transcription factors (PIF3) that regulate genes of the phenylpropanoid pathway. Therefore, it is possible that photoperiod, via the *E3* gene, can regulate the metabolism of secondary compounds, including phenolic compounds.

In the exudates of the neutral-day line L71-920 under short-day conditions, a significant decrease in monosaccharides, phenolics, flavonoids, and soluble proteins was observed. At the same time, amino acid and oligosaccharide contents increased, though total soluble carbohydrate levels did not. The L71-920 genotype has all *e1*, *e2*, *e3* genes in a recessive state, defining its neutral-day type. Thus, these trends, differing from other lines, may result from the allelic state of the *E*-genes. Additionally, under short-day conditions, L71-920 exudates stimulated *B. japonicum* 634b growth – unlike other lines. Moreover, these exudates most strongly promoted rhizobial biofilm formation.

A trophic-signaling model of photoperiodic regulation of plant-bacteria interactions. The obtained results, confirm the hypothesis that amino acids (nitrogen sources) are crucial for rhizobial growth and biofilm formation. Indeed, amino acids have chemoattractant properties – e.g., aspartate and glutamate are strong attractants for *B. japonicum* (Barbour et al., 1991; Althabegoiti et al., 2008). Chuiko et al. (2002) also showed high chemoattractant activity from phenylalanine, glutamic acid, and threonine toward *B. japonicum* 634b. Soluble carbohydrates appear not to affect rhizobial growth or biofilm formation. Literature data suggest that few carbohydrates act as attractants for *B. japonicum* – notably arabinose, sucrose, and mannitol (a sugar alcohol) (Barbour et al., 1991; Chuiko et al., 2002). Conversely, monosaccharides significantly influence *Bacillus* biofilm formation, both alone and in co-culture with rhizobia. Increases in monosaccharides under short-day conditions in all isolines except L71-920 positively correlated with enhanced *Bacillus* biofilm formation. Meanwhile, decreased monosaccharides in L71-920 exudates inhibited biofilm formation by *B. subtilis*, alone and with rhizobia. The impact of phenols and flavonoids on rhizobial growth and biofilm formation is interesting. For most lines (except L63-3117), these compounds limited or inhibited rhizobial growth and biofilm formation. Generally, flavonoids induce nod-gene expression in microsymbionts. However, genistein, daidzein, daidzin, luteolin, and naringenin – known nod inducers – do not show chemoattractant properties (Kape et al., 1991; Barbour et al., 1991). Furthermore, at high concentrations, both phenols and flavonoids can inhibit biofilm formation (Lee et al., 2012). Thus, photoperiod is a crucial factor influencing the qualitative and quantitative composition of root exudates. Considering the differential photoperiod effects on short-day and neutral-day plants, the photoperiod sensitivity genes (*E*-genes) play a significant role.

Thus, our results, along with the analysis of literature data, allow us to propose a hypothetical trophic-signaling model of photoperiodic regulation in the interaction between soybean and soil bacteria (Fig. 3).

We suggest that the photoperiod (short/long) interacts with the soybean genotype – particularly the photoperiod sensitivity genes *E1*, *E2*, and *E3* – which in turn regulates plant metabolic processes and alters the metabolic profile of root exudates. These changes are genotype-specific, as different *E1-E3* alleles lead to distinct effects. Root exudates contain both trophic and signaling compounds that create the rhizosphere nutrient environment and modulate plant–bacteria interactions. The trophic factors influence soil bacteria in various ways: for example, amino acids promote *B. japonicum* growth and biofilm formation, while monosaccharides stimulate biofilm development in *B. subtilis*, both in monoculture and during co-cultivation with rhizobia. Interbacterial interactions in the rhizosphere are important. In our study, biofilm formation under long-day conditions was not observed when the bacteria were cultured separately, but biofilm formed during their co-cultivation. Signaling molecules such as flavonoids have a modulatory effect: they suppressed *B. japonicum* growth in cv. Clark under short-day conditions, while promoting biofilm formation in isolines L63-3117. This model highlights the dual role of root exudates as trophic and signaling agents modulated by photoperiod and soybean genotype in influencing plant-bacteria interactions in the rhizosphere.

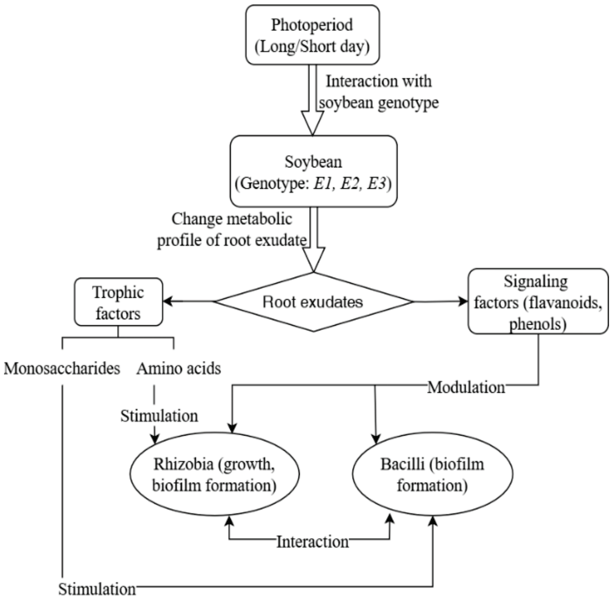

Fig. 3. A trophic-signaling model of photoperiodic regulation of soybean-soil bacteria interactions; photoperiod (short/long day) modulates soybean metabolism via *E*-genes (*E1*, *E2*, *E3*) changing the composition of root exudates; trophic factors (amino acids, monosaccharides) and signaling factors (flavonoids, phenolics) differentially affect *Bradyrhizobium japonicum* and *Bacillus subtilis*

Conclusion

The study revealed a significant role of photoperiod in determining the qualitative and quantitative composition of soybean root exudates and their influence on symbiotic interactions with rhizobacteria. It was established that genotype-specific sensitivity of soybean to photoperiod, determined by the allelic state of *E*-genes, modulates root secretion of carbohydrates, proteins, amino acids, phenolic compounds, and flavonoids, which affect the growth and biofilm formation of *B. japonicum* and *B. subtilis*. A short photoperiod (9 hours) promoted the accumulation of monosaccharides and a decrease in amino acids in the exudates of most lines, whereas the day-neutral line L71-920 showed an increase in amino acids and a decrease in monosaccharides in its exudates, which stimulated rhizobial growth. Flavonoids, the accumulation of which depended on the presence of a dominant state of the *E3* gene, mainly exhibited an inhibitory effect on rhizobial growth, emphasizing their complex regulatory role in plant-microorganism interactions. At the same time, for *B. subtilis*, the short photoperiod promoted biofilm formation when using exudates of lines that have at least one of the *E1–E3* genes in the dominant state, whereas exudates of line L71-920 inhibited biofilm formation by bacilli both in monoculture and during co-cultivation with rhizobia. It is likely that such trends are determined by the accumulation of monosaccharides in root exudates. The obtained results deepen our understanding of the influence of photoperiod and plant sensitivity to it on the metabolic processes that determine the quantitative and qualitative composition of root exudates and, accordingly, the indirect impact on the control of rhizosphere microbiota. This allows us to suggest the existence of a trophic-signaling module of photoperiodic regulation of rhizosphere microbiota.

Additionally, the results of the study may be useful for the selection of soybean cultivars with different photoperiod sensitivities to ensure effective interactions with rhizosphere microbiota in various latitudes; for the development of bioinoculants based on *B. japonicum* and *B. subtilis*, specific to certain soybean varieties and cultivation conditions; and for improving the agrotechnologies of soybean cultivation in different latitudinal zones.

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The authors declare no conflict of interest.

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