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Stimulation of Acidic Reduction of Nitrite to Nitric Oxide by Soybean Phenolics: Possible Relevance to Gastrointestinal Host Defense

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ABSTRACT: This study aimed to evaluate the potential of soybean-promoted acidic nitrite reduction and to correlate this activity with the content of phenolics and with the bactericidal activity against *Escherichia coli* O157:H7. Extracts of embrionary axes and cotyledons enriched in phenolics increased •NO formation at acidic pH at values that were 7.1 and 4.5 times higher, respectively, when compared to the reduction of the nonenriched extracts. Among the various phenolics accumulated in the soybean extracts, five stimulated nitrite reduction in the following decreasing order of potency: epicatechin gallate, chlorogenic acid, caffeic acid, galic acid and *p*-coumaric acid. Extracts of embrionary axes presented higher contents of epicatechin gallate and caffeic acid, compared to that of cotyledons, indicating a positive correlation between activity of the extracts and content of phenolics with regard to nitrite reducing activity. Soybean extracts enriched in phenolics interacted synergistically with acidified nitrite to prevent *E. coli* O157:H7 growth. The results suggest that soybean phenolics may interfere with the metabolism of •NO in an acidic environment by accelerating the reduction of nitrite, with a potential antimicrobial effect in the stomach.

KEYWORDS: nitric oxide, nitrous acid reduction, phenolics, soybean, bactericidal effect, *Escherichia coli* O157:H7

INTRODUCTION

The generation of the radical nitric oxide (•NO) in animal cells results mainly from the action of a family of enzymes named nitric oxide synthases (NOS), which catalyze the synthesis of •NO from the oxidation of the amino acid L-arginine into L-citrulline.[1] The generated NO plays a role in a large number of metabolic functions and is involved in the development of various pathophysiological processes.[1] As a radical with a short half-life in biological systems, most of •NO produced in the body is rapidly oxidized to nitrite/nitrate, which are molecules that were, until recently, considered inert for human metabolism. However, more recent evidence suggests that these anions are important forms of storage of •NO, which can be activated to generate the radical under certain conditions.[2] Thus, •NO can be formed from nitrite/nitrate reduction through mechanisms independent of NOS, such as catalysis by heme proteins, xanthine oxidoreductase and thiol-containing enzymes, particularly under hypoxic/anoxic conditions such as those occurring during myocardial infarction and stroke.[2] The acidic reduction of nitrite has also been recently considered to be a nonenzymatic mechanism of physiological relevance for the generation of •NO in the gastrointestinal tract.[3]

A diet rich in vegetables also results in a large ingestion of inorganic nitrate, which is found in large quantities within vegetables. Although the metabolism of dietary nitrate was long thought to generate nitrosamines with potentially carcinogenic effects, a clear link between nitrate intake and gastric cancer in humans has not been definitively established.[4] Instead, more recent studies have highlighted the important biological functions of the nitrate−nitrite−•NO pathway and discussed the potential use of nitrate and nitrite for novel •NO-based therapeutics.[4,5]

The inorganic nitrate obtained from the diet is secreted in saliva and reduced to nitrite by bacterial microbiota. In the acidic pH of the stomach, nitrite is converted into nitrous acid, which can spontaneously generate •NO through a chemical univalent reduction along with other nitrogen oxides.[3,6] •NO produced from the reduction of nitrite in gastric juice may exert multiple beneficial effects on human health, protecting against gastrointestinal diseases and increasing gastric motility, mucosal blood flow and mucus thickness (see ref 5).

The reduction of nitrite in acidic medium is a very slow reaction that can be accelerated by the presence of endogenous reducing agents, such as thiocyanate and ascorbic acid.[7] Thus, reducing agents with appropriate reduction potentials may facilitate the reduction of nitrous acid, thereby generating •NO.

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Indeed, it has been demonstrated that dietary phenolics that are present in fruits, vegetables and beverages have appropriate redox potentials to act as reducing agents of acidified nitrite, thus facilitating •NO production under the acidic conditions of the stomach.[8−10] The broad activity of the flavonoid quercetin in promoting the reduction of nitrite in acidic pH was related to its molecular skeleton, specifically to the hydroxyl group at carbon position 3 in the central ring.[8] Peri et al.[9] have shown that polyphenols of apple extracts were responsible for increasing •NO release caused by acidification of saliva, chlorogenic acid and catechin being the most active and concentrated species. They also reported that following oxidation, the *o*-semiquinone radicals are nitrated, decreasing the concentration of more reactive nitrogen species, which are also produced following nitrous acid reduction, such as $^{\bullet}NO_2$, N_2O_3 and ONOOH.[6] Phenolics present in red wine were also suggested to cooperate with ascorbic acid in the reduction of nitrite to •NO in the stomach, inducing smooth muscle relaxation.[11] Takahama and Hirota[12] have reported that when coffee is ingested, in addition to chlorogenic acid and its isomers, melanoidins could also react with salivary nitrite and thiocyanate in the gastric lumen to produce •NO.

Rocha et al.[13] have recently demonstrated that, in the presence of nitrite, phenol-containing dietary products induce a significant increase in the amount of •NO in the stomach. They measured the amount of •NO in the air expelled from the stomach of healthy volunteers following a meal containing polyphenols. Their data shows that polyphenols from diverse dietary sources that are endowed with distinct chemical structures interact with nitrite at pH 2 in the stomach, reducing it univalently to •NO via the concomitant formation of a phenol phenoxyl radical (Ph−O•) intermediate, as previously proposed.[9,10] Furthermore, selected mixtures of polyphenols and nitrite induce structure-dependent muscle relaxation in stomach strips, suggesting that the polyphenols consumed in dietary products may exert a biological impact as a local relaxant.[13]

The health benefits of a diet enriched in fruits and vegetables has been frequently attributed to their phenolic compounds, which would positively interfere with metabolism due to their antioxidant activity and contribute to the prevention of many degenerative diseases.[14] This is also true in the case of soybeans, for which beneficial effects on human health have been attributed mostly to the antioxidant and estrogenic activities of the isoflavones.[15] However, the concentration of phenolic compounds that can reach body tissues following dietary intake is frequently much lower than the values that have proven efficient to test antioxidant activity in *in vitro* assays due to an extensive metabolism and a normally low absorption of plant phenolics in the gastrointestinal tract.[16] In light of new findings about the effect of plant phenolics in promoting acidic reduction of nitrite, it has been suggested that part of the beneficial effects on human health of a diet rich in vegetables may also result from the action of its phenolics in facilitating the production of •NO in the acidic pH of the stomach.[8−10]

In order to investigate the potential of foods containing soybean products interfering with the generation of •NO at the gastrointestinal tract, we investigated the acidic nitrite reducing activity of extracts prepared from embrionary axes and cotyledons of soybean seeds in the present study. It has previously been shown that treatment of soybean cotyledons with the inducing agent sodium nitroprusside was able to activate the phenylpropanoid pathway and cause exudation of large amounts of free isoflavones in the medium.[17] In addition to the isoflavones, activation of the phenylpropanoid pathway in soybeans may also lead to accumulation of other phenolics[18] that have been identified as facilitators of the acid reduction of nitrite. In this study, a treatment with sodium nitroprusside was used to correlate the phenolic content in soybean seed extracts with acidic nitrite reduction activity. To show that this activity may have physiological significance, we evaluated the effect of the soybean extracts with acidified nitrite on the growth of *Escherichia coli* O157:H7, a food-borne pathogen that can cause sporadic cases of diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in humans.[19]

MATERIALS AND METHODS

Chemicals. Methanol, formic acid and acetone were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Sodium nitroprusside, *S*-nitroso-*N*-acetyl-penicillamine, $CuCl_2$ and Folin−Ciocalteu were obtained from Sigma (St. Louis, MO). The following standard compounds used as references were purchased from several sources: *p*-coumaric acid, gallic acid, hydroxybenzoic acid, tartaric acid, daidzein, genistein (Sigma-Aldrich, St. Louis, MO); glycitein, daidzin, genistin, glycitin (LC Laboratories, Woburn, MA); caffeic acid, chlorogenic acid (Alexis Biochemicals, San Diego, CA); benzoic acid (Amresco, Inc., Solon, OH) and epicatechin gallate (Wako Chemicals USA, Inc., Richmond, VA). The remaining chemicals were obtained from Merck (Darmstadt, Germany).

Preparation of Soybean Extracts Enriched in Phenolics. Soybean (*Glycine max* (L.) Merr) seeds of the cultivar IAC-18 (kindly provided by Nelson R. Braga from the Agronomic Institute of Campinas, Campinas, São Paulo, Brazil) were treated with sodium nitroprusside to increase the total phenolic content.[17] Soybean seeds were first sterilized by soaking in 0.3% commercial sodium hypochlorite for 10 min and washed thoroughly three times with distilled water. Seed coats were removed, discarded and detached; embrionary axes or cotyledons were treated with an aqueous solution (50 units/10 mL) of 100 mM sodium nitroprusside or incubated in water (control). Samples were stirred (100 rpm) in an Erlenmeyer flask in the dark at 25 °C for 20 h. After the treatment, phenolic compounds were extracted from cotyledons or embrionary axes with their exudates (50 units in 10 mL) with 20 mL of acetone/water (50:50 v/v) for 12 h under agitation, according to Xu et al.[20] Subsequently, the suspension was centrifuged at 3000 rpm for 15 min, and the supernatant was collected, dried and stored at −20 °C for further analysis.

Analysis of the Total Phenolic Content in Soybean Extracts. Total phenolic content in soybean extracts was determined using the Folin−Ciocalteau reagent.[21] The soybean extracts (10 μL) were mixed with 50 μL of diluted Folin−Ciocalteau reagent (1:10). After the reaction solution had been incubated at room temperature for 5 min, 40 μL of a 4% $CaCO_3$ solution was added and mixed thoroughly; the mixture was incubated at room temperature for 120 min. The absorbance of the solution was then determined at 740 nm. Quercetin and gallic acid were used as standard references.

Identification of the Phenolic Compounds in Soybean Extracts by Ultrahigh Performance Liquid Chromatography−Mass Spectrometry (UPLC−MS). The analysis was carried out in a UPLC−MS TQD Micromass (Waters, Manchester, England) equipped with an ultratorque binary pump system, a column oven, an automatic injection system, and a TQD mass spectrometer. The experimental conditions for separation, identification and quantification followed those described by Hvattum[22] with modifications. The column used was a 50 mm × 2.1 mm i.d., 5 μm, Waters C18. Phenolic compounds were separated using a gradient of 0.5% formic acid (A) and a 99.5% methanol/0.5% formic acid (v/v) (B). The gradient consisted of 99% A for 2 min, followed by 95% A for 1 min, 80% A for 3 min, 40% A for 3 min, 20% A for 1 min, 5% A for 1.4 min, and finally,

reconditioning for 2.2 min with 99% A. The complete chromatographic run took 14 min, the flow rate was 0.2 mL/min and the column temperature was set to 30 °C. After chromatographic separation, the eluate was ionized by electrospray (ESI) interface and analyzed in the negative ion mode under the following conditions: a capillary voltage of −3000 V, a cone voltage of −30 V and a scan range from m/z 100 to 800; the desolvation gas temperature was set to 300 °C, and the source temperature was 150 °C. The phenolic compounds were identified by comparison with the retention time and mass spectrum of authentic standards and quantified by UPLC−MS, using external calibration curves for gallic acid, chlorogenic acid, tartaric acid and epicatechin gallate.

Electrochemical Determination of •NO Concentration. •NO concentrations were measured electrochemically at 25 °C using the ISO-NOP electrode connected to a free radical analyzer Apollo 4000 (World Precision Instruments, Sarasota, FL) according to Zhang,[23] with modifications. Aliquots of the soybean extracts and the standards of flavonoids and other phenolic compounds were added to the reaction medium (1 mL) in a closed chamber NOCHM-4 (World Precision Instruments), under constant agitation. To determine the nitrite reduction, the reaction medium was composed of 0.1 M KCl/HCl (pH 2.0), 0.1 M citrate phosphate buffer (pH 3.5) or 0.1 M phosphate buffer (pH 5.0 or 7.0). The experiments were initiated by nitrite addition to the reaction medium and then by addition of aliquots of the extracts or standards. The •NO electrode was calibrated with *S*-nitroso-*N*-acetylpenicillamine in 0.1 M $CuCl_2$.

Determination of •NO Concentration by Chemiluminescence. •NO concentrations were measured by chemiluminescence at 25 °C using a chemiluminescence nitric oxide analyzer NOA280 (Sievers Instruments, Boulder, CO) according to Bonini et al.,[24] with modifications. The nitrite (50 to 200 μM) and 50 μL extracts (35 μg dry weight/μL) were directly injected into a vessel containing 5 mL of reaction medium (0.1 M KCl/HCl solution, pH 2.0) and maintained under N_2 flow. After 10 min, the total •NO produced from nitrite reduction was carried on the stream of nitrogen into the chemiluminescence detector. The peak areas of the samples were calculated with the instrument software and compared with those of standard solutions (different volumes of a •NO saturated solution (1.75 nmol/μL)) analyzed under the same experimental conditions. The saturated •NO solution was prepared by bubbling pure •NO gas (Linde Gases, São Paulo, Brazil) for 15 min through Ar-purged phosphate buffer (10 mM, pH 7.2) in a rubber-sealed vial.

Effects of Soybean Extracts and Acidified Nitrite on Bacterial Growth. The pathogenic *Escherichia coli* strain O157:H7 (obtained from the Collection of Bacterial Virulence Factors Laboratory of the Department of Genetics and Evolution and Bioagents, State University of Campinas, Campinas, SP, Brazil) was cultured on tryptone soy agar plates and subsequently incubated in tryptone soy broth medium (pH 7.2) for 24 h at 37 °C to yield 1×10^7 colony-forming units (CFU)/mL on the day of the experiments. Bacterial suspensions were centrifuged at 7300*g* for 15 min. The supernatant was then discarded and the bacterial cells were added to sterile 2 mL Eppendorf tubes containing 1 mL of reaction medium to obtain a final density of 1×10^7 bacteria/mL. The reaction medium was composed of 0.1 M KCl/HCl (pH 2.0) or 0.1 M phosphate buffer (pH 7.0) alone or with nitrite (50 μM) and soybean extracts (350 μg). The tubes were then incubated for 5 min, after which 100 μL of each sample was removed, serially diluted (1:10) with saline (0.9%) solution and transferred to selective MacConkey medium agar plates, according to the procedure of Miles and Misra[25] with modifications. Previous tests had established the plating dilutions necessary to yield numbers of colonies adequate for counting. For each treatment, an aliquot of 25 μL from a 10^{-6} dilution of the *E. coli* culture was plated as a drop (6 drops per plate). Plates were then incubated at 37 °C and the colonies were counted after 24 h. The experiments were performed in triplicate.

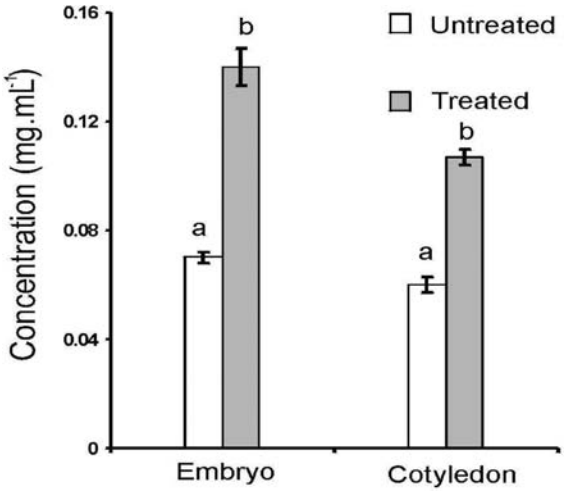

Figure 1. Concentration of total phenolics in soybean extracts: effect of sodium nitroprusside treatment. Embrionary axes (embryo) or cotyledons of soybean seeds were incubated with an eliciting solution of sodium nitroprusside. In the control (untreated) the tissues were incubated in water. The data represent the mean ± SD of three independent experiments ($p < 0.001$).

Statistical Analysis. The results were expressed as the mean ± standard deviation of experiments ($n = 3-5$). Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by Tukey's test with $P < 0.05$ indicating significance.

RESULTS AND DISCUSSION

Preparation of Phenolic-Enriched Soybean Extracts. To increase the concentration of phenolics, soybean seeds were treated with the inducing agent sodium nitroprusside.[17] Methanol extracts prepared from detached embryonic axes and cotyledons were then analyzed for total phenolic content. As shown in Figure 1, treatment with sodium nitroprusside increased the number of total phenolics compared to untreated extracts. In the case of the embrionary axis extracts, this increase was doubled from 0.7 mg/mL of total phenolics in untreated extracts to 1.4 mg/mL in the treated extracts. The concentration of total phenolics in cotyledon extracts was also increased from 0.6 mg/mL in untreated extracts to 1.1 mg/mL in the treated extracts. The results also show that the amount of total phenolics in embrionary axis extracts is higher than in cotyledon extracts (mg/mL).

Nitrite-Dependent NO Formation by Soybean Extracts. The capacity of soybean extracts and the effect of the enrichment of the extracts in phenolic compounds in promoting the formation of •NO from acidified nitrite were evaluated using a specific electrode. First, the effect of $NaNO_2$ concentration on the formation of •NO was analyzed under experimental conditions outlined in this study. After 5 min of incubation in 1 mL of 0.1 M KCl/HCl (pH 2.0), 25 μM nitrite produced approximately 0.12 nmol of •NO, which increased to 0.24 nmol in the presence of 50 μM nitrite. In the presence of 75 μM and 100 μM nitrite, total •NO formation was further increased to 0.34 nmol and 0.44 nmol, respectively. These results show that •NO production was directly proportional to nitrite concentration in the experimental conditions chosen for this study.

Soybean extracts were then evaluated for their capacity to stimulate acid reduction of nitrite; this activity was compared with the flavonoids quercetin and luteolin, which were previously

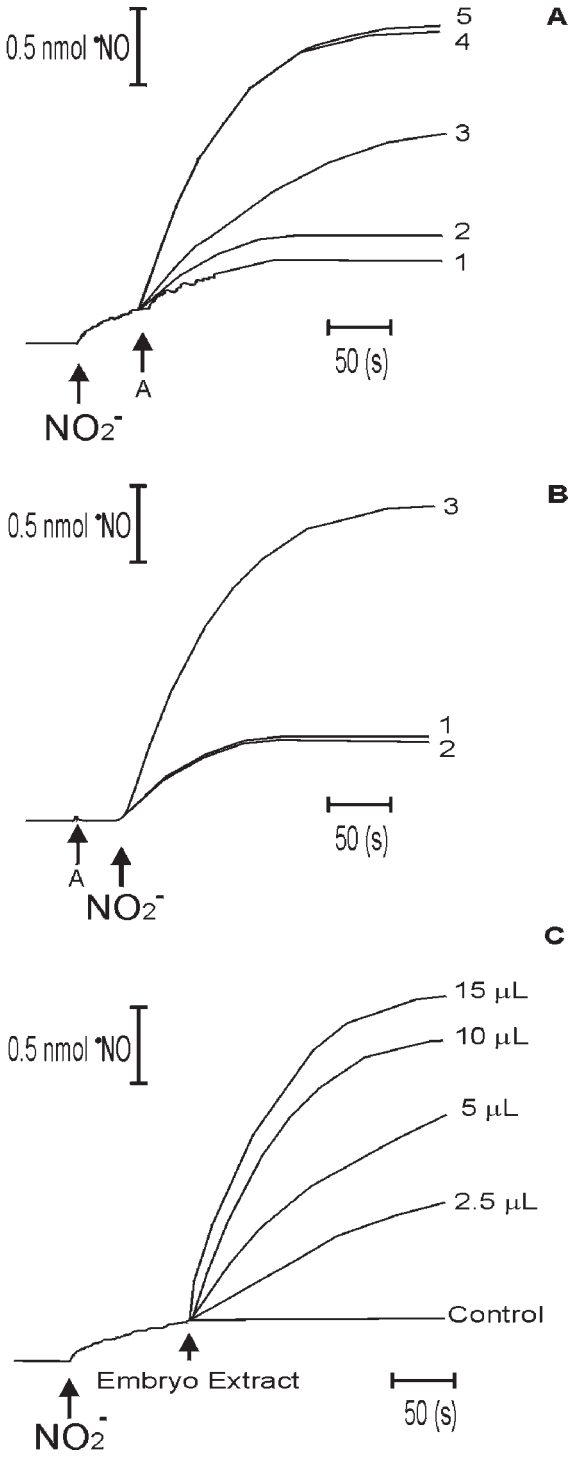

Figure 2. Effect of soybean extracts on acidic reduction of nitrite measured electrochemically. Extracts or isolated flavonoids were added in 1 mL of reaction medium (pH 2.0) and nitrite (50 μM). (A) Nitrite was added before the extracts or isolated flavonoids: no further addition (1); 25 μM luteolin (2); 10 μL (35 μg/mL) of cotyledon (3) or embrionary axis (4) extracts enriched in phenolic compounds and 25 μM quercetin (5). (B) Nitrite was added after the extracts: nitrite alone (1), 10 μL (35 μg/mL) of embrionary axis extracts unenriched (2) and enriched (3) in phenolic compounds. (C) Dose curve of $^{\bullet}$NO formation promoted by enriched extracts of embrionary axis (embryo extract) in the presence of 50 μM nitrite.

identified as facilitators of the acid reduction of nitrite.[8] As shown in Figure 2A, 10 μL (350 μg) of phenolic-enriched extracts from embrionary axes and cotyledons increased nitrite reduction leading to the production of 1.4 and 0.9 nmol of $^{\bullet}$NO at pH 2.0; these values were 7.0 and 4.5 times higher, respectively, when compared to the reduction of untreated extracts, which produced 0.2 nmol of $^{\bullet}$NO, as shown in Figure 2B. Figure 2B also shows that, when the extracts are added to the acid solution alone, there is no $^{\bullet}$NO signal; the radical is produced only after the addition of nitrite. These results show that the effect of the extracts on $^{\bullet}$NO production is dependent on the presence of nitrite. To prove that the sodium nitroprusside would not cause a false positive result, an elicitor solution (100 mM) was extracted similarly to the soybean extracts and evaluated for its ability to reduce nitrite. The time course of $^{\bullet}$NO formation induced by the sodium nitroprusside extract was not significantly different from that induced by nitrite alone (not shown). The flavonoids quercetin and luteolin, at 25 μM, significantly increased the spontaneous nitrite reduction resulting in the production of 1.42 and 0.3 nmol of $^{\bullet}$NO, respectively, after a 5 min incubation. This lower activity of luteolin compared to quercetin is consistent with previous results,[7] which related the effectiveness of flavonoids, in terms of the reduction of nitrous acid, to their molecular structure. The influence of extract concentration on nitrite reduction was evaluated and is shown in Figure 2C. Production of $^{\bullet}$NO ranged from 0.8 nmol (2.5 μL extract) to 1.89 nmol (15 μL extract). Volumes greater than 15 μL of extract show no significant difference in nitrite reduction activity, suggesting that there was a saturation of nitrite concentration used in the medium or that there was a steady-state concentration due to reaction of $^{\bullet}$NO with oxygen, forming the radical $^{\bullet}NO_2$, which can react with the extract components.

Chemiluminescence Detection of Nitrite-Dependent $^{\bullet}$NO Formation. The nitrite reducing activity of soybean extracts identified by electrochemical analysis was confirmed by the detection of $^{\bullet}$NO using chemiluminescence. First, the influence of nitrite concentration on the kinetics of $^{\bullet}$NO generation was evaluated. As expected, increasing the nitrite concentration caused a proportional increase in $^{\bullet}$NO formation. The spontaneous reduction of nitrite after 10 min in 5 mL of a reaction medium at pH 2.0 produced 1.6 nmol of $^{\bullet}$NO with 50 μM nitrite (Figure 3) and increased to 9.7 nmol of $^{\bullet}$NO in the presence of 200 μM nitrite (not shown). The enriched extracts from the embrionary axes and cotyledons stimulated the nitrite (50 μM) reduction, and, after 10 min, 5.7 and 3.7 nmol of $^{\bullet}$NO were formed, respectively, whereas luteolin (50 μM) produced 4.4 nmol of $^{\bullet}$NO (Figure 3). These results showed a significant effect on the acidic reduction of nitrite compared to control (nitrite only) and untreated extracts from embrionary axes and cotyledons, which produced 0.79 and 0.83 nmol of $^{\bullet}$NO, respectively. Thus, the nitrite reduction activity of the extracts from embrionary axes and cotyledons was increased by 7.2 times and 4.4 times after phenolic enrichment. Figure 3 also shows that there was no reductive activity of the elicitor solution, indicating again that the induction assay used in soybean tissues was responsible for the reductive activity observed in the extracts. Additionally, the amount of $^{\bullet}$NO generated in the presence of untreated extracts (~0.81 nmol) was lower than the control (1.6 nmol of $^{\bullet}$NO) and $^{\bullet}$NO was not detected when treated or untreated extracts were incubated in the absence of nitrite.

Because chemiluminescence is performed in an anaerobic environment, it reduces the interference of oxygen. $^{\bullet}$NO

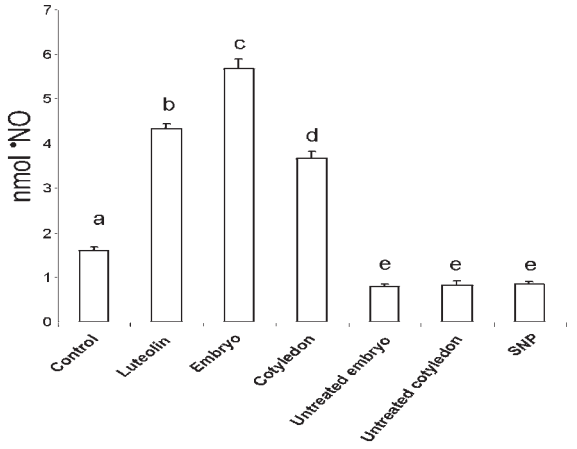

Figure 3. Effect of soybean extracts on acidic reduction of nitrite measured by chemiluminescence. The total •NO produced was determined after 10 min of nitrite addition (50 μM) in 5 mL of reaction medium (pH 2.0) containing only reaction medium (control) or luteolin (50 μM) or 50 μL of embrionary axis extract (35 μg dry weight /μL) or 50 μL of cotyledons extract (35 μg dry weight /μL) treated or untreated with sodium nitroprusside (SNP) or 50 μL of a sodium nitroprusside solution (100 mM), as indicated. The data represent the mean ± SD of three independent experiments ($p < 0.01$).

generated in aerobiosis reacts with molecular oxygen to form other nitrogen oxides,[26] which explains why total •NO detected by the chemiluminescence method was higher compared to the electrochemical measurement. However, by both analyses, the extracts of the embrionary axis showed the best reducing activity when compared to the cotyledon or to extracts that were not treated with sodium nitroprusside.

Effect of pH on the Nitrite Reducing Activity of Soybean Extracts. The influence of pH on the reduction of nitrite was evaluated electrochemically and by chemiluminescence. As shown in Table 1, enriched extracts from embrionary axes and cotyledons at pH 3.5 produced approximately 0.87 and 0.66 nmol of •NO, respectively, in the presence of 50 μM nitrite, as measured by the chemiluminescence assay. These values are 6.5 and 5.6 times lower than those found at pH 2.0, respectively. The treated extracts from embrionary axes and cotyledons showed approximate 2- to 3-fold reductions compared to untreated extracts (0.35 and 0.34 nmol of •NO, respectively) at pH 3.5. Similarly, when comparing the electrochemical detection data, the increase in pH from 2.0 to 3.5 decreased the nitrite reducing activity of enriched extracts from embrionary axes and cotyledons by 5.2 and 3.6 times, respectively. These activities were 3.8 and 3.1 times higher compared to the sodium nitroprusside untreated extracts. The activities of the flavonoids quercetin and luteolin were also evaluated for comparison at pH 3.5, and formation of •NO was sharply reduced, as measured by both techniques (Table 1). Additionally, when the experiments presented in Table 1 were carried out using 0.1 M phosphate buffer at pH 5.0 and 7.0, •NO production was not detected (results not shown). Overall, these results show that the nitrite reducing activities are directly related to the acidity of the medium (Table 1). Additionally, there is a good correlation between the values from chemiluminescence and electrochemistry. Thus, at pH 2.0, at which point the nitrite reducing activity was maximized, •NO production by extracts of the embrionary axis and cotyledon was increased after enrichment with phenolics by 7.0-fold (from 0.2 to 1.4 nmol) and 7.2-fold (from 0.79 to 5.67 nmol) and by 4.5-fold (from 0.2 to 0.9 nmol) and 4.4-fold (from 0.83 to 3.68 nmol), as determined electrochemically or by chemiluminescence, respectively. Also, compared with the activity of the nitrite-only (controls) at pH 2.0, quercetin increased •NO production by 10-fold (from 0.25 to 2.5 nmol) and 10.2-fold (from 1.59 to 16.23 nmol) and luteolin by 2.0-fold (from 0.25 to 0.5 nmol) and 2.76-fold (from 1.59 to 4.39 nmol) when determined electrochemically and by chemiluminescence, respectively.

Table 1. Effect of pH on the Nitrite Reducing Activity of Soybean Extracts Determined by Chemiluminescence and with an •NO Electrode

	nmol of •NO			
	chemiluminescence		electrochemistry	
pH	2.0	3.5	2.0	3.5
control	1.59	0.44	0.25	0.09
quercetin	16.23	1.06	2.5	0.3
luteolin	4.39	0.62	0.5	0.01
embryo[a]	5.67	0.87	1.4	0.27
cotyledon	3.68	0.66	0.9	0.25
untreated embryo[a]	0.79	0.35	0.2	0.07
untreated cotyledon	0.83	0.34	0.2	0.08

[a] Embrionary axis.

Identification of the Phenolics in Soybean Extracts and Correlation with the Nitrite Reducing Activity. Phenolic compounds present in soybean extracts from embrionary axes and cotyledons (treated or untreated with sodium nitroprusside) were identified by UPLC−MS. Based on standard references, it was possible to identify the following phenolic compounds for the elicitor-treated extracts: epicatechin gallate, benzoic acid, hydroxybenzoic acid, chlorogenic acid, gallic acid, caffeic acid, *p*-coumaric acid and tartaric acid. In the untreated extracts, traces of these phenolics were detected, except for *p*-coumaric acid, which was present in treated and untreated extracts. Additionally, as previously demonstrated,[17] large amounts of the aglycon forms of isoflavones, i.e., daidzein, genistein and glycitein, accumulated in sodium nitroprusside-treated extracts; in the untreated extracts, these isoflavones were mostly in their malonyl- and glycosyl-β-conjugated forms (results not shown).

The phenolics identified in the extracts were then evaluated for their capacity to reduce acidified nitrite using commercial standards. Among all the phenolic compounds identified in the extracts, five showed activity: epicatechin gallate was the most effective, followed by chlorogenic acid; gallic acid and cafeic acid showed intermediate activity, and *p*-coumaric acid showed low activity when the rates of •NO formation were compared to rates promoted by epicatechin gallate (Figure 4). The reduction of nitrite in the presence of tartaric acid or hydroxybenzoic acid was not significantly different from that in the presence of only nitrite (Figure 4). Still, the free- or β-conjugated isoflavones had no effect on acidic reduction of nitrite (results not shown). The potential of phenolic compounds to reduce nitrite at acidic pH has been related to their molecular structures, specifically the number of reactive hydroxy groups, molecular steric factors and the ability to form a semiquinone[8−10,13] Thus, the high nitrite reducing activity of epicatechin gallate could be due to the following factors: the galloyl group easily generates a semiquinone, there

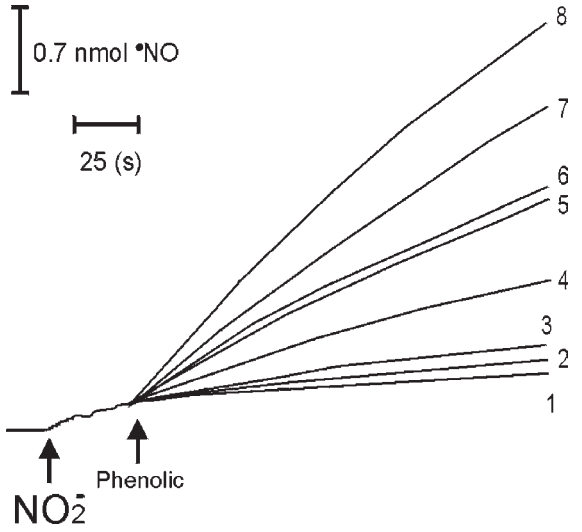

Figure 4. Time courses of •NO formation from acidified nitrite promoted by phenolics identified in the soybean extracts. The •NO production was measured electrochemically at pH 2.0, after addition of 50 μM nitrite (1) and 50 μM of tartaric acid (2), hydroxybenzoic acid (3), *p*-coumaric acid (4), caffeic acid (5), gallic acid (6), chlorogenic acid (7) and epicatechin gallate (8).

Table 2. Concentration of Phenolic Compounds in Soybean-Enriched Extracts with Acidic Nitrite Reduction Activity[a]

	μg of phenolic/g of dry extract			
	SNP		control	
extracts	E	C	E	C
gallic acid	70	69	trace	trace
chlorogenic acid	60	60	trace	trace
caffeic acid	70	62	nd	trace
epicatechin gallate	200	186	nd	nd

[a] SNP, sodium nitroprusside; E, embrionary axis; C, cotyledon; nd, not detected.

is a phenolic coupling and acidic pH induces changes in the resonance of the molecule.[27]

Besides showing the best ability to reduce nitrite among those identified in soybean extracts (Figure 4), the epicatechin gallate was the phenolic compound accumulated to the highest concentration in the treated soybean extracts, which was 200 μg/g and 186 μg/g of dry extract from embrionary axes and cotyledons, respectively (Table 2). Caffeic acid was accumulated in higher amounts in treated embrionary axis extracts (70 μg/g) compared to cotyledon extracts (62 μg/g), whereas equivalent amounts of gallic (70 μg/g) and chlorogenic (60 μg/g) acids accumulated in both treated extracts (Table 2). These results suggest that the best effect observed for embrionary axis extracts in the acidic reduction of nitrite was due to higher levels of epicatechin gallate and caffeic acid. Thus, it was possible to observe a positive correlation between the effect of extracts in acid reduction of nitrite and amount of phenolic compounds with nitrite reducing activity.

Although the nitrite reducing activity of soybean extracts was detected only after treatment of the seed parts with sodium

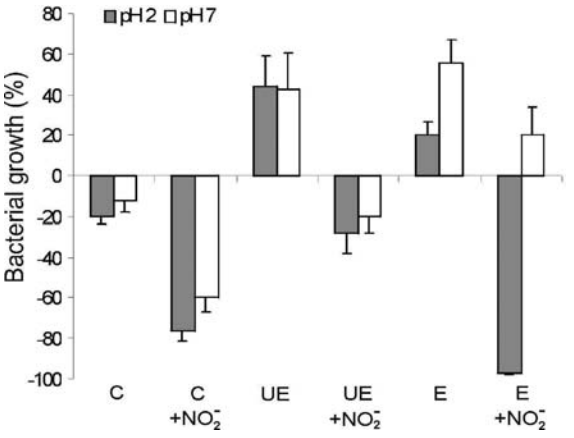

Figure 5. Effect of pH, nitrite and soybean extracts on *Escherichia coli* O157:H7 growth. Bacterial suspensions were incubated at pH 2.0 or 7.0 alone (C) or in the presence of 50 μM nitrite ($+NO_2^-$) and embrionary axis extracts (350 μg) either untreated (UE) or treated (E) with sodium nitroprusside. The data show the results of two independent experiments performed in triplicate.

nitroprusside, the possibility that soybeans *in natura* may have this activity cannot be excluded because the phenolics accumulated in the extracts were mostly released from the plant tissues. This effect of sodium nitroprusside was previously attributed to •NO released from its structure,[17] which increases transcription of phenylalanine ammonia-lyase and chalcone synthase,[28] the initial enzymes in the phenylpropanoid pathway. However, we cannot rule out the possibility that sodium nitroprusside could react with phenols to yield nitroso derivatives that could in turn function as •NO donors. However, mass spectrometry (measuring the *m*/*z* values of protonated and deprotonated ions of the phenolic compounds present in the soybean extracts) did not identify any molecular compound that might indicate the formation of such products (not shown). Nevertheless, the incubation of extracts in acid medium alone was not sufficient to generate any •NO signal, which occurred only after the addition of nitrite (Figure 2). Because the action of •NO released from sodium nitroprusside occurs in the early steps of the phenylpropanoid pathway, it is most likely that there are no more •NO or other reactive nitrogen intermediates to react with the phenolics produced, whose significant accumulation occurs only after 20 h of treatment with the donor •NO.[17]

Bactericidal Effects of Soybean Extracts and Acidified Nitrite. The growth of *E. coli* O157:H7 was evaluated after exposure to the reaction medium at pH 2.0 or 7.0 and in the presence of nitrite alone or in combination with the soybean extracts. As shown in Figure 5, exposure of bacterial suspensions for 5 min to the reaction medium at pH 2.0 or 7.0 had only a slight effect on bacterial growth, decreasing cell counts by 20% and 12%, respectively, in relation to the bacterial starting inoculum (10^{-6} dilution). Inhibition of *E. coli* O157:H7 growth was significantly accentuated by the addition of 50 μM nitrite (cell counts were decreased by 76% at pH 2 and 60% at pH 7), indicating a bactericidal effect independent of the pH. Untreated extracts from embrionary axes had a stimulatory effect on bacterial growth (increasing cell counts by 43%), which could result from the fact that this bacterium is normally cultivated in a soy culture medium. However, when nitrite was mixed with untreated extracts from embrionary axes, the nitrite bactericidal

effect was observed at both pH values, as seen by growth inhibition of 28% and 20% at pH 2.0 and 7.0, respectively. These results indicate that untreated embrionary axes alone have no bactericidal effects. Incubation of the bacterial suspensions with enriched extracts alone had a positive effect on cell counts, and this effect was higher at pH 7.0 (55%) than at pH 2.0 (20%). However, when extracts from embrionary axes enriched in phenolics were mixed with nitrite, a drastic inhibition on bacterial growth was observed when the incubation was carried out at pH 2.0 (97% inhibition, already seen at the 10^{-5} dilution) but not at pH 7.0 (20% growth stimulation). These results indicate that *E. coli* O157:H7 was killed after exposure to an acidified mixture of nitrite and soybean extract enriched in phenolics.

Various reports have shown that addition of nitrite to acidified solutions increases the antimicrobial effect against various pathogens, including enterobacter, yeast and *Helicobacter*.[3,29,30] Accordingly, as shown in Figure 5, nitrite inhibited bacterial growth in our experimental conditions. However, when soybean extracts enriched in phenolics were mixed with acidified nitrite, the bactericidal effect was drastically increased, showing that soybean extracts enriched in phenolics synergistically interact with nitrite to prevent *E. coli* O157:H7 growth in an acidic environment. This bactericidal effect could result from the stimulated production of $^{\bullet}$NO that occurs under these circumstances (Figures 2 and 3, Table 1). It is possible that the beneficial effects of soybean to human health are greater than the antioxidant and estrogenic activities of their isoflavones.[15] Benefits could also be related to the reduction of dietary nitrites, including those used for food preservation.[4]

Overall, our results suggest that soybean phenolics may interfere with the metabolism of $^{\bullet}$NO by accelerating the reduction of acidified nitrite, with a potential antimicrobial activity that may increase the host defense in the gastrointestinal tract.

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REFERENCES

(1) Moncada, S.; Palmer, R. M.; Higgs, E. A. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **1991**, *43*, 109–142.

(2) Gladwin, M. T.; Schechter, A. N.; Kim-Shapiro, D. B.; Patel, R. P.; Hogg, N.; Shiva, S.; Cannon, R. O., 3rd; Kelm, M.; Wink, D. A.; Espey, M. G.; Oldfield, E. H.; Pluta, R. M.; Freeman, B. A.; Lancaster, J. R., Jr; Feelisch, M.; Lundberg, J. O. The emerging biology of the nitrite anion. *Nat. Chem. Biol.* **2005**, *1*, 308–314.

(3) Benjamin, N.; O'Driscoll, F.; Dougall, H.; Duncan, C.; Smith, L.; Golden, M.; McKenzie, H. Stomach NO synthesis. *Nature* **1994**, *368*, 502.

(4) Milkowski, A.; Garg, H. K.; Coughlin, J. R.; Bryan, N. S. Nutritional epidemiology in the context of nitric oxide biology: A risk-benefit evaluation for dietary nitrite and nitrate. *Nitric Oxide* **2010**, *22*, 110–119.

(5) Lundberg, J. O.; Weitzberg, E.; Gladwin, M. T. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Discovery* **2008**, *7*, 156–167.

(6) McKnight, G. M.; Smith, L. M.; Drummond, R. S.; Duncan, C. W.; Golden, M.; Benjamin, N. Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut* **1997**, *40*, 211–214.

(7) Moriya, A.; Grant, J.; Mowat, C.; Williams, C.; Carswell, A.; Preston, T.; Anderson, S.; Iijima, K.; McColl, K. E. In vitro studies indicate that acid catalysed generation of N-nitrosocompounds from dietary nitrate will be maximal at the gastro-oesophageal junction and cardia. *Scand. J. Gastroenterol.* **2002**, *37*, 253–261.

(8) Takahama, U.; Oniki, T.; Hirota, S. Oxidation of quercetin by salivary components. Quercetin-dependent reduction of salivary nitrite under acidic conditions producing nitric oxide. *J. Agric. Food Chem.* **2002**, *50*, 4317–4422.

(9) Peri, L.; Pietraforte, D.; Scorza, G.; Napolitano, A.; Fogliano, V.; Minetti, M. Apples increase nitric oxide production by human saliva at the acidic pH of the stomach: a new biological function for polyphenols with a catechol group? *Free Radical Biol Med.* **2005**, *39*, 668–681.

(10) Gago, B.; Lundberg, J. O.; Barbosa, R. M.; Laranjinha, J. Red wine-dependent reduction of nitrite to nitric oxide in the stomach. *Free Radical Biol. Med.* **2007**, *43*, 1233–1242.

(11) Rocha, B. S.; Gago, B.; Barbosa, R. M.; Laranjinha, J. Diffusion of nitric oxide through the gastric wall upon reduction of nitrite by red wine: Physiological impact. *Nitric Oxide Biol. Chem.* **2010**, *22*, 235–241.

(12) Takahama, U.; Hirota, S. Reduction of nitrous acid to nitric oxide by coffee melanoidins and enhancement of the reduction by thiocyanate: Possibility of its occurrence in the stomach. *J. Agric. Food Chem.* **2008**, *56*, 4736–4744.

(13) Rocha, B. S.; Gago, B.; Barbosa, R. M.; Laranjinha, J. Dietary polyphenols generate nitric oxide from nitrite in the stomach and induce smooth muscle relaxation. *Toxicology* **2009**, *265*, 41–48.

(14) Havsteen, B. H. The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther.* **2002**, *96*, 67–202.

(15) McCue, P.; Shetty, K. Health benefits of soy isoflavonoids and strategies for enhancement: a review. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 361–367.

(16) Halliwell, B.; Zhao, K.; Whiteman, M. The gastrointestinal tract: a major site of antioxidant action? *Free Radical Res* **2001**, *33*, 819–830.

(17) Modolo, L. V.; Cunha, F. Q.; Braga, M. R.; Salgado, I. Nitric oxide synthase-mediated phytoalexin accumulation in soybean cotyledons in response to the *Diaporthe phaseolorum* f. sp. *meridionalis* elicitor. *Plant Physiol.* **2002**, *130*, 1288–1297.

(18) Ferrer, J. L.; Austin, M. B.; Stewart, C., Jr; Noel, J. P. Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. *Plant Physiol. Biochem.* **2008**, *46*, 356–370.

(19) Johannes, L.; Römer, W. Shiga toxins--from cell biology to biomedical applications. *Nat. Rev. Microbiol.* **2010**, *8*, 105–16.

(20) Xu, G.; Ye, X.; Chen, J.; Liu, D. Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract. *J. Agric. Food Chem.* **2007**, *55*, 330–335.

(21) Sun, T.; Hou, J.; Hu, J.; Chen, S.; Cai, X.; Zou, G. Chemical composition, cytotoxic and antioxidant activity of the leaf essential oil of *Photinia serrulata*. *Food Chem.* **2007**, *103*, 355–358.

(22) Hvattum, E. Determination of phenolic compounds in rose hip *(Rosa canina)* using liquid chromatography coupled to electrospray ionization tandem mass spectrometry and diode-array detection. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 655–662.

(23) Zhang, X. Real time and in vivo monitoring of nitric oxide by electrochemical sensors - from dream to reality. *Front. Biosci.* **2004**, *9*, 3434–3446.

(24) Bonini, M. G.; Miyamoto, S.; Di Mascio, P.; Augusto, O. Production of the carbonate radical anion during xanthine oxidase turnover in the presence of bicarbonate. *J. Biol. Chem.* **2004**, *279*, 51836–51843.

(25) Miles, A. A.; Misra, S. S. The estimation of the bactericidal power of the blood. *J. Hygiene* **1938**, *38*, 732–749.

(26) Amano, F.; Noda, T. Improved detection of nitric oxide radical ($NO^{\bullet}$) production in an activated macrophage culture with a radical scavenger, carboxy PTIO and Griess reagent. *FEBS Lett.* **1995**, *368*, 425–428.

(27) Bors, W.; Foo, L. Y.; Hertkorn, N.; Michel, C.; Stettmaier, K. Chemical studies of proanthocyanidins and hydrolyzable tannins. *Antioxid. Redox. Signal.* **2001**, *3*, 995–1008.

(28) Delledonne, M.; Xia, Y.; Dixon, R. A.; Lamb, C. Nitric oxide functions as a signal in plant disease resistance. *Nature* **1998**, *394*, 585–588.

(29) Dykhuizen, R. S.; Frazer, R; Duncan, C.; Smith, C. C.; Golden, M.; Benjamin, N.; Leifert, C. Antimicrobial effect of acidified nitrite on gut pathogens: importance of dietary nitrate in host defense. *Antimicrob. Agents Chemother.* **1996**, *40*, 1422–1425.

(30) Dykhuizen, R. S.; Frazer, R.; McKenzie, H.; Golden, M.; Leifert, C.; Benjamin, N. Helicobacter pylori is killed by nitrite under acidic conditions. *Gut* **1998**, *42*, 334–337.