TERRA LATINOAMERICANA

Folic Acid (vitamin B9) or Inoculation with Beneficial Microbes Enhances Plant Growth in *Phaseolus vulgaris* L.
Efectos del Ácido fólico (vitamina B9) o de la Inoculación de Microbios Benéficos Incrementa el Crecimiento de *Phaseolus vulgaris* L.

Jacob Bañuelos[1], Esperanza Martínez-Romero[3], Noé Manuel Montaño[2‡], and Sara Lucía Camargo-Ricalde[2]

[1] Universidad Autónoma Metropolitana, Doctorado en Ciencias Biológicas y de la Salud. Ciudad de México, México; (J.B.).
[2] Universidad Autónoma Metropolitana, Unidad Iztapalapa, Departamento de Biología, División de Ciencias Biológicas y de la Salud. Av. San Rafael Atlixco No. 186, Col. Vicentina. 09340 Alcaldía Iztapalapa, Ciudad de México, México; (N.M.M.), (S.L.C.R.).
‡ Corresponding author: nmma@xanum.uam.mx
[3] Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México. Av. Universidad 2001, Col. Chamilpa. 62210, Cuernavaca, Morelos, Mexico; (E.M.R.).

SUMMARY

Folic acid (vitamin B9) plays a crucial role in chlorophyll synthesis, and its exogenous application to plants could positively influence the Dark Green Color Index (DGCI) and plant growth. This study investigated how vitamin B9 supplementation could affect the growth of inoculated *Phaseolus vulgaris* L. (Fabaceae) and its symbiont. The inoculant used contained both arbuscular mycorrhizal fungi (AMF) and bacteria. We measured DGCI in leaves, plant growth variables and AMF colonization 35 days after seeding. Our results showed that in *P. vulgaris* treated with vitamin B9, both plant growth and DGCI increased compared to controls. However, the addition of vitamin B9 to *P. vulgaris* plants, inoculated with a mixture of AM fungi and bacteria, did not further increase plant growth, suggesting that inoculated plants are not limited by vitamin B9. This study is the first to highlight the role of vitamin B9 in inoculated *P. vulgaris* plants. Independently, both vitamin B9 amendment and inoculation with beneficial microbes increased the measured variables; though, their combined effect was not additive. Therefore, our results suggest that the addition of vitamin B9 does not further increase the measured variables when the plants are inoculated with a mixed AMF and bacterial inoculant.

Index words: *arbuscular mycorrhizal fungi, B9, chlorophyll*, *proline*.

RESUMEN

El ácido fólico (vitamina B9) juega un papel crucial en la síntesis de clorofila y su aplicación exógena a las plantas podría influir positivamente en el índice de verdes obscuros (DGCI) y crecimiento vegetal. Este estudio investigó cómo la suplementación de la vitamina B9 puede afectar el crecimiento de plantas de *Phaseolus vulgaris* L. (Fabaceae) inoculadas y al simbionte. El inoculante utilizado contenía tanto hongos micorrízicos arbusculares (HMA) como bacterias. Se midió el Índice de Verdes Obscuros (IVO) en las hojas, variables de crecimiento de las plantas y la colonización de HMA, 35 días después de la siembra. Los resultados mostraron que en *P. vulgaris* tratadas con la vitamina B9, tanto el crecimiento de la planta como el índice de verdes obscuros aumentaron en comparación con el control. Sin embargo, la adición de la vitamina B9 a las plantas de *P. vulgaris* inoculadas con una mezcla de HMA, no aumentó el crecimiento de estas, lo que sugiere que las plantas inoculadas no están limitadas por la vitamina B9. Este estudio es el primero en destacar el efecto de la adición de la vitamina B9 en plantas de *P. vulgaris* inoculadas con organismos benéficos en variables de crecimiento. De manera independiente, tanto la adición de la vitamina B9 como del inoculante incrementaron las variables medidas; sin

Cita recomendada:

Bañuelos, J., Martínez-Romero, E., Montaño, N. M., & Camargo-Ricalde, S. L. (2025). Folic Acid (vitamin B9) or Inoculation with Beneficial Microbes Enhances Plant Growth in *Phaseolus vulgaris* L. *Terra Latinoamericana, 43*, 1-10. e2090. https://doi.org/10.28940/terra.v43i.2090

Received: September 25, 2024.
Accepted: December 16, 2024.
Article, Volume 43.
April 2025.

Editor de Sección:
Dr. Luis Alonso Valdez Aguilar

Editor Técnico:
M.C. Ayenia Carolina Rosales Nieblas

Copyright: © 2025 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY NC ND) License (https://creativecommons.org/licenses/by-nc-nd/4.0/).

embargo, el efecto combinado no fue aditivo. Por lo tanto, nuestros resultados sugieren que la adición de la vitamina B9 no produce un mayor incremento en las variables medidas cuando las plantas están inoculadas con un inoculante mixto de HMA y bacterias.

Palabras clave: *hongos micorrízicos arbusculares, B9, clorofila, prolina.*

INTRODUCTION

Folates are a group of water-soluble B vitamins that play a crucial role in various biological processes, including plant growth and development. They are essential for the formation of purines (Zrenner, Sitt, Sonnewald, and Boldt, 2006), which are critical for cell division, and they influence on the production of plant hormones such as auxins (Li *et al*., 2023) and ethylene (Wang, Li, and Ecker, 2002); as well as root formation and cell elongation (Hwang *et al.*, 2018). Folates also enhance stress tolerance in plants (Aljuaid *et al*., 2022; Alsamadany, Mansour, Elkelish, and Ibrahim, 2022), they are necessary for methionine, serine and glycine formation (Basset, Quinlivan, Gregory, and Hanson, 2005), and act as regulators during symbiotic interactions (Bekuzarova, Kozyrev, Shabanova, Lushenko, and Weissfeld, 2020; Banuelos, Martínez-Romero, Montaño, and Camargo, 2021; Piya *et al*., 2023). Although plants can synthesize folates, they often rely on external sources for an adequate supply. This has been observed in *Phaseolus vulgaris* L. (Fabaceae), where foliar folic acid (vitamin B9) application (0.1-0.2 mM) significantly increased shoot and root growth, as well as the content of chlorophyll *a*, *b* and carotenoids (Alsamadany *et al.*, 2022). Additionally, the folate precursor pABA (4-aminobenzoic acid) enhanced plant growth, particularly by increasing the number of nitrogen-fixing bacteria root nodules in *Trifolium rapens* L. (Faboideae) and *Medicago sativa* L. (Fabaceae) (Bekuzarova *et al.*, 2020). Folate synthesis increases during elevated light exposure (Jabrin, Ravanel, Gambonnet, Douce y Rébeillé, 2003), as folates are involved in chlorophyll biosynthesis through their contribution to one-carbon metabolism (Van-Wilder *et al.*, 2009). Accordingly, an increase in folate supply could alter chlorophyll production, but its effect on arbuscular mycorrhizal fungi (AMF) symbiosis is still unknown.

Folates also confer tolerance against plant stress, primarily through regulating plant metabolites. One of these molecules is proline, an amino acid that serves as an osmoregulator for salt and drought stress; and varies during symbiosis with AMF (Bañuelos *et al*., 2012). Proline is an indicator not only of stress conditions but is connected to several other cellular processes, such as energy status, nutrient availability, and changes in redox balance and its catabolism is linked to photosynthesis and mitochondrial respiration (Alvarez, Savouré, and Szabados, 2022). Therefore, it is an important marker of general plant health and metabolic status. Although folates are essential vitamins for most living organisms, AMF does not biosynthesize most vitamins (Ghignone *et al*., 2012). Therefore, the addition of folates may affect root colonization by AMF and host plant responses. Other plant root symbionts, like *Rhizobium*, are affected by vitamin supplementation or increase (Yang, Bhuvaneswari, Joseph, King, and Phillips, 2002), and as AMF share a common symbiosis signaling pathway (De Brujin, 2019), this symbiosis also might be affected. The effect of different vitamins has been studied in *P. vulgaris* (Keshavarz y Moghadam, 2017; Banuelos, Martínez, Montaño, and Camargo, 2023) that not only form a symbiosis with rhizobia but also with AMF. We hypothesize that the addition of an external source of stable folate, like vitamin B9, might increase the leaf's DGCI while affecting AMF root colonization or their effect on the plant. The objectives of this study were to determine if the exogenous application of vitamin B9 could change the AMF effect in *Phaseolus vulgari*s L. (Fabaceae), as well as the growth and the Dark Green Color Index (DGCI) of *P. vulgaris* L. leaves.

MATERIALS AND METHODS

Experimental Design

The experimental design was completely randomized, using a 2×2 factorial scheme with two inoculation treatments (with and without AMF), two vitamin irrigation treatments (with and without vitamin B9), and a combination of both. We considered one plant as an experimental unit. We used 4 replicates per treatment, yielding 16 experimental units.

Experiment Management Conditions

The experiment was conducted for 35 days under greenhouse conditions before flowering, at the Faculty of Agricultural Sciences, Universidad Veracruzana, Xalapa, México. The greenhouse maintained an average relative humidity of 74% and an average temperature of 20 °C. The plants were grown in one-liter pots and irrigated every other day until the harvest. The substrate used for the study was obtained from the top 10 cm of soil in a coffee-growing area located within a mountain mesophyll forest in Coatepec, Veracruz, Mexico. Soil analysis was performed by the Soil Analysis Laboratory of the National Institute of Ecology (INECOL), Xalapa, Mexico, and presented the following physical and chemical characteristics: 63.5% phosphorus (P) removed (Blakemore); 40.52 mg kg^{-1} of available P (PO_4^-, Olsen); 34.28 mg kg^{-1} ammonium (NH_4-N) (salicylate method); 36.28 mg kg^{-1} nitrates (NO_3-N); 1016 mg kg^{-1} pyrophosphate-extractable iron (Fe) (PeF); 7.13% organic matter (OM), (Walkley-Black); 4.14% organic carbon (OC) (Walkley-Black); 667.71 mg kg^{-1} sodium (Na^+) (atomic absorption spectrometry); 693.38 mg kg^{-1} calcium (Ca^{2+}) (atomic absorption spectrometry), and 794.77 mg kg^{-1} magnesium (Mg^{2+}) (atomic absorption spectrometry). The pH_{H2O} was 6.7, obtained in a suspension of soil and deionized water (1:2 w/v). The substrate was sterilized for 40 min by autoclaving, at 120 °C, 1.05 kg cm^{-2} (Khavazi, Rejali, Seguin, and Miransari, 2007; Sheteiwy *et al*., 2022).

Plant Material

"Negro jamapa" is a cultivar of *P. vulgaris* L., a bean variety from Veracruz, México (Voysest, 1983). Negro Jamapa bean seeds were surface disinfected by treating them with 70% ethanol for 1 min, followed by 1.2% Na^+hypochlorite for 15 min. The seeds were then rinsed four times with sterilized water, once with 2% Na^+thiosulfate and a final rinsing with water (Rosenblueth and Martínez, 2004). Seeds were germinated in sterilized quartz sand at total water-holding capacity (42.7%) before being planted in 1 L pots (13 cm diameter) with autoclaved soil (120 °C, 1.05 kg cm^{-2} for 40 min).

Folic Acid (vitamin B9) Supply

We used folic acid, a fully oxidized monoglutamate form of vitamin B9, for this experiment. A stock solution of 50 µM vitamin B9 from Sigma-Aldrich (BioReagent for cell culture) was prepared in deionized sterile water by stirring for 10 min in the dark and stored at 4 °C until use. We added 50 mL of vitamin B9 to the plants, 2 cm from the stem, at night to prevent degradation, five days after mycorrhizal inoculation and every five days until harvest. The control treatments were irrigated with sterile deionized water in the same manner.

Arbuscular Mycorrhizal Fungi (AMF) Inoculum

The inoculum was provided by the Laboratory of Beneficial Organisms, Faculty of Agricultural Sciences, Universidad Veracruzana, Mexico. This inoculant contained 15 species of AMF (Table 1) (Banuelos *et al.,* 2023) and diverse bacterial species (Hernández-Álvarez, Peimbert, Rodrìguez, Trejo, and Alcaraz, 2023). The inoculum consisted of spores (6500 per 100 g) and propagules within *Brachiaria decumbens* Stapf (Poaceae) roots (87% AMF colonization), with sand as a vehicle. The inoculum was added (10 g) in the transplantation hole below the plantlet. After 35 days, we measured the AMF colonization percentage (Giovannetti and Mosse, 1980) by clearing and staining plant roots (Phillips y Hayman, 1970).

The inoculum was produced in host plants and contains mainly AMF, however, there are associated bacteria in this inoculant (Hernández-Álvarez *et al.*, 2023).

Dark Green Color Index (DGCI)

We used an indirect method to measure leaf nitrogen (N) content as an average of three fully developed leaves at half the height of the plant. This is a non-destructive method that allows us to use an Android mobile phone camera to measure changes in the leaves by obtaining a DGCI on a scale from 0-1 (Petiole LTD, 2019). DGCI is a relative quantitative measure of greenness, closely related to leaf N concentration (Rorie, Purcell, Karcher, and King, 2011a; Kaler *et al.,* 2020). The procedure relies on a calibration pad to normalize the color among samples, while normalizing the background light changes. The mobile phone was placed on a stationary stand, 5 cm above the leaf sample, in a controlled light environment and a picture was taken to then be used in the Petiole Pro mobile phone application.

Table 1. List of the 14 species of arbuscular mycorrhizal fungi (AMF) contained in the inoculum mixture added to *Phaseolus vulgaris* L. (Fabaceae) plants.

Family	AMF species	Synonym
Archaeosporaceae	*Archaeospora trappei* (R.N. Ames and Linderman) J.B. Morton and D. Redecker	*Acaulospora trappei*
Acaulosporaceae	*Acaulospora sp*	
Gigasporaceae	*Dentiscutata savannicola* (R.A. Herrera and Ferrer) C. Walker and A. Schüßler	*Gigaspora savannicola; Fuscutata savannicola; Scutellospora savannicola*
	Gigaspora rosea T.H. Nicolson and N.C. Schenck	
	Cetraspora pellucida (T.H. Nicolson and N.C. Schenck) Oehl, F.A. Souza and Sieverding	*Scutellospora pellucida*
Diversisporaceae	*Diversispora varaderana* J. Błaszkowski, G. Chwat, Kovács and Góralska	
Claroideoglomeraceae	*Claroideoglomus etunicatum* (W.N. Becker and Gerd.) C. Walker and A. Schüßler	*Glomus etunicatum; Entrophospora etunicata*
Glomeraceae	*Dominikia bernensis* Oehl, Palenz., Sánchez-Castro, N.M.F. Sousa and G.A. Silva	
	Dominikia lithuanica J. Błaszkowski, G. Chwat, A. Góralska	
	Rhizophagus aggregatus (N.C. Schenck and G.S. Sm.) C. Walker	*Glomus aggregatum. Rhizoglomus aggregatum*
	Glomus macrocarpum T.H. Nicolson and Gerd	*Endogone macrocarpa*
	Funneliformis mosseae T.H. Nicolson and Gerd	
	Kamienskia bistrata J. Błaszkowski, G. Chwat and Kovács	
	Rhizophagus irregularis Błaszk., Wubet, Renker and Buscot (C. Walker and A. Schüßler comb. Nov)	
	Rhizophagus neocaledonicus D. Redecker, Crossay and Cilia	

Taken from Bañuelos *et al*. (2023).

An affordable leaf N content meter is commonly used to determine leaf greenness and indirectly infer leaf chlorophyll concentration (Kaler *et al*., 2020). Karcher and Richardson (2003) found that digital image analysis can be used to determine differences among plants fertilized with N by measuring their DGCI. Also, Rorie *et al*. (2011b) demonstrated that the coefficient of determination (R^2) between DGCI and leaf N concentration (g 100 g^{-1}) was 0.81-0.91 and that the DGCI is an effective color-image analysis to assess N concentration in leaves (Rhezali, Prucell, Roberts, and Greub, 2018).

Proline Content Determination

Proline content was determined using the method of Bates, Waldren, and Teare (1973), with modifications. A 0.25 g sample of all the leaves was homogenized with liquid N in a mortar, and 5 mL of 3% sulfosalicylic acid was added. The extract was filtered under a vacuum. Aliquots of 0.5 mL of the extract were mixed with 0.5 mL of acid ninhydrin solution, together with 0.5 mL of acetic acid. Samples were heated for 30 min at 90 °C and cooled in an ice bath until room temperature. Toluene was used to extract the amino acid, and samples were incubated for 20 min in a dark environment. The absorbance of the organic phase was measured at 520 nm using a spectrophotometer (Thermo-scientific GENESYS 10S UV-Vis). Proline concentration was calculated using a calibration curve and expressed as micromoles per miligram.

Plant Growth Variables

Harvested plants were divided into root and shoot and then oven-dried at 45 °C for three days until reaching a constant weight. We counted the number of trifoliate leaves at harvest. The height of the plant was measured from the base of the stalk at harvest, and the trifoliate leaves were counted manually. Leaf area was measured using the mobile app) Petiole Pro (Petiole LTD, 2019).

Statistical Analysis

A two-way ANOVA was performed to compare treatments. Results were expressed as means with standard deviation (±). Significant differences between treatment means were analysed using Tukey's post hoc test (HSD, $P \leq 0.05$). Normality and homogeneity of variances were addressed using Shapiro–Wilk and Levene's test. All data analyses were performed in Minitab® 18.1 (Minitab, 2017).

RESULTS AND DISCUSSION

Plant Growth

As *P. vulgaris* production moves towards more sustainable production methods, there is a need for novel information regarding the use of biofertilizers and non-synthetic metabolites. In this study, we observed that the addition of the inoculant increased the leaf area significantly (ANOVA, F:13.63, $P \leq 0.044$), whereas vitamin B9 significantly increased DGCI (ANOVA, F:19.27, $P \leq 0.001$) (Figure 1, 2). The combination of both factors showed significant differences in plant height (F:13.63, $P \leq 0.004$), number of trifoliate leaves (F:17.36, $P \leq 0.002$) and leaf area (F:7.74, $P \leq 0.018$). Specifically, we observed a 67% increase in shoot dry weight with the addition of vitamin B9 (F: 5.01, $P \leq 0.047$; Figure 1a). This aligns with previous reports in *Capsicum annuum* L. (Solanaceae) and *Triticum aestivum* L. (Poaceae), where vitamin B9 supplementation also led to biomass increase (Al-Said and Kamal, 2008; Esfandiari, Enayati, Sabaghina, and Janmohammadi, 2012). This increase could potentially translate into higher yields at later stages, as has been suggested in other studies (Rashidi, Yousefi, Pouryusef, and Goicoechea, 2021). Vitamin B9 is essential for processes, such as DNA synthesis, cell division, and amino acid metabolism, all of which play crucial roles in supporting overall plant growth (Gorelova, Ambach, Rébeillé, Stove, and Straeten, 2017). Its affordability and proven effectiveness in crops such as *Pisum sativum* L. (Fabaceae) (Farouk and Abdul-Qados, 2018) and *P. vulgaris* (Ibrahim, Ibrahim, and El-Gawad, 2021) position it as a promising tool for improving crop productivity. The positive impact of vitamin B9 supplementation on plant weight indicates that it likely acts as a general growth promoter, contributing to enhanced biomass accumulation in both shoot and root tissues (Burguieres, McCue, Kwon, and Shetty, 2007). Other studies have shown that vitamin B9 deficiency can cause growth abnormalities and reduce overall plant vigour (Li *et al*., 2023), and by increasing its supply, we can raise its vigour. The increase in plant weight upon vitamin B9 addition could also be attributed to the restoration of folate-related metabolic pathways, leading to improved growth and development (Ayala-Rodríguez, Barrera, Ruiz, and López, 2017). Folates are essential for pyruvate biosynthesis, which is vital for biomass production (Bar-Even, Noor, Flamholz, and Milo, 2013; Kim *et al.*, 2020). Recently, Llerena-Ramos *et al*. (2025). used beneficial microorganisms such as *Azotobacter chroococum* and *Metarhizium anisopliae*, in combination with organic fertilizers such as K_4SiO_4-enriched liquid compost in rice to improve sustainability and productivity, concluding that, these approaches not only promote higher crop yields by reducing panicle sterility, but also support the overall health of the agricultural ecosystem, which is crucial for the future of global agriculture.

The inoculum significantly increased leaf area (F: 4.36, $P \leq 0.030$; Figure 1b), which is consistent with the well-documented role of AMF in nutrient acquisition and transport (Augé Stodola, Ebel, and Duan, 1995; Kavanova, Grimoldi, Lattanzi, and Schnyder, 2006). The expansion of leaf area may be due to the improved nutrient availability facilitated by the mycorrhizal hyphal network, which enhances photosynthetic capacity and overall plant vigour (Cela *et al*., 2022; Tao, Dong, Wang, Chen, and Tang, 2022) or by the effect of the associated bacteria (Mashatleh *et al.,* 2024). AMF increased plant height (F: 5.25, $P \leq 0.017$); however, when vitamin B9 was added, the inoculated plants had lower height compared to uninoculated ones (F: 13.63, $P \leq 0.0.004$; Figure 1c). Additionally, plants showed an increase in the number of trifoliate leaves when inoculated (F: 5.92, $P \leq 0.012$), but the number of trifoliate leaves did not increase when vitamin B9 was added (F: 0.02, $P \leq 0.888$, Figure 1d). Interestingly, the absence of an additive effect of vitamin B9 supplementation in AMF-bacteria inoculated plants on these variables, suggests a potential threshold in folate availability, where additional folate does not confer extra benefits. The plant might already optimize vitamin B9 acquisition to a point where the AMF inoculation does not further enhance the physiological processes responsible for plant growth parameters. Moreover, the regulatory mechanisms governing plant development might involve other factors, such as hormonal signaling (Pozo, López, Azcón, and García, 2015). The lack of an additive effect could also indicate that the specific interaction between

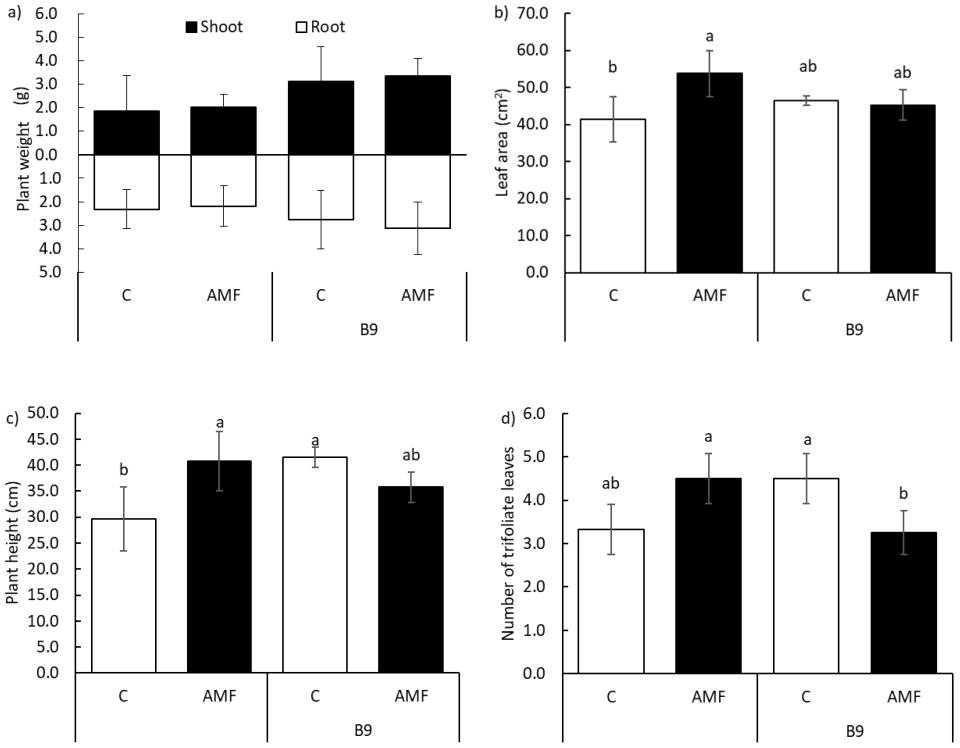

Figure 1. a) *Phaseolus vulgaris* L. dry weight (foliar and root), b) leaf area, c) plant height, and d) the number of trifoliate leaves in inoculated plants (AMF) and not inoculated controls (C) and irrigated with 50 µM of folic acid (vitamin B9) or not (control, C); 35 days after mycorrhizal and bacterial mixed inoculation. Means ± standard deviation with n=4. Different letters above bars indicate significant differences (Tukey HSD *P* ≤ 0.05).

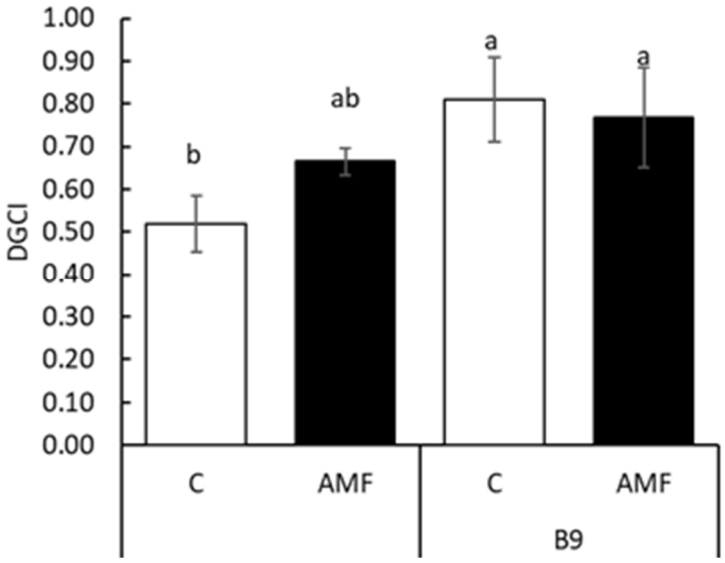

Figure 2. Dark Green Color Index (DGCI) in *Phaseolus vulgaris* L. inoculated (AMF) and non-inoculated controls (C) and irrigated with 50 µM folic acid (vitamin B9), or not (control, C) 35 days after mycorrhizal and bacterial mixed inoculation. Means ± standard deviation with n=4. Different letters above bars indicate significant differences (Tukey HSD *P* ≤ 0.05).

microbes and the host plant modulates the response to exogenous vitamin B9 differently compared to non-mycorrhizal plants (Seddas *et al*., 2009). The bacteria present in the inoculum could also be a source of vitamins, in particular B9 (Hernández-Álvarez, *et al.,* 2023), but we found no differences between inoculated plants and inoculated plus vitamin B9 plants (Figure 1). We also measured AMF colonization in all treatments but found no significant differences between them, with no colonization in non-AMF treatments and 25.75±8.5% for the AMF treatment and 21±6.1% for the AMF+B9 treatment.

Dark Green Color Index (DGCI)

We measured the DGCI as an indicator of the leaf N content affected by the inoculant and vitamin B9 supplementation. Inoculated plants did not increase the DGCI (F: 1.31, $P \leq 0.276$). Usually, AMF inoculation increases chlorophyll content in plants which is related to leaf N content (Kavanova *et al*., 2006; Kayoumu *et al*., 2023); although studies with AMF inoculated *Vitis vinifera* L. (Vitaceae) have also shown that chlorophyll content does not vary compared to uninoculated plants (Ye, Wang, and Li, 2022). Irrigation with vitamin B9 increased DGCI (F:19.27, $P \leq 0.001$); whereas the combination of vitamin B9 plus inoculant did not differ from the vitamin B9 treatment alone (F:4.40, $P \leq 0.0.06$) (Figure 2). The increase in DGCI by vitamin B9 supplementation could be due to folates being involved in N metabolism, mediating its translocation from the root to the shoot (Banuelos *et al*., 2021). The N availability affects plants' photosynthesis as chlorophyll is associated with leaf N content (Wang *et al*., 2021), and the involvement of vitamin B9 in N translocation could have caused the increase in DGCI.

Proline Content

The inoculation reduced proline content in leaves, although the reduction was not significant (F: 4.02, $P \leq 0.07$). On the other hand, we observed that vitamin B9 addition significantly decreased the concentration of the metabolite proline, both in control and in AMF-inoculated plants (F: 37.63, $P \leq 0.000$; Figure 3). This decrease in proline could be related to the improved water-use efficiency observed with vitamin B9 treatment, which has been reported to reduce the need for osmoregulatory metabolites like proline in plants (Ibrahim *et al*., 2021; Al-Elwany *et al.,* 2022). For instance, foliar irrigation with 150 µM of vitamin B9 can increase water usage efficiency by 29% (Ibrahim *et al*., 2021). In *P. vulgaris*, vitamin B9 supplementation has been shown to alleviate salt stress, which is often linked to water use efficiency (Alsamadany *et al*., 2022; Ma, Wei, Liu, Liu, and Liu, 2021). Therefore, the growth-promoting effects of vitamin B9 may also contribute to overall plant fitness, as reflected in lower proline levels.

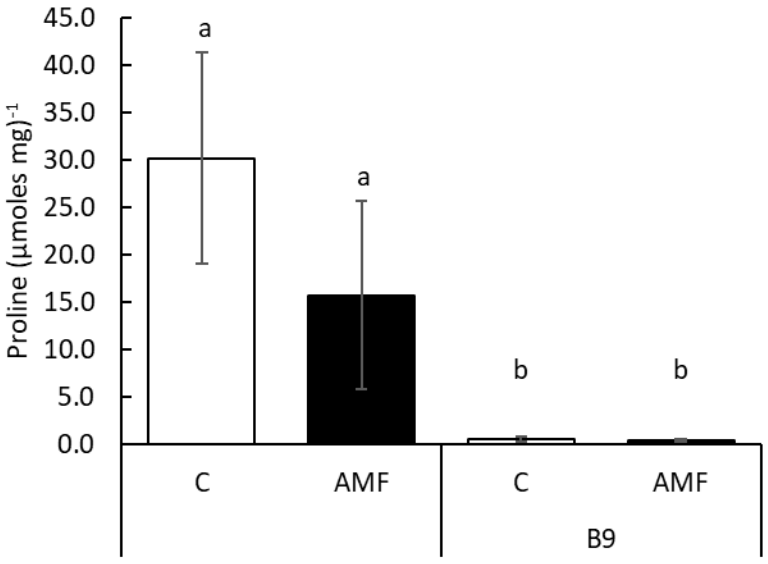

Figure 3. Proline content in leaves of inoculated (AMF) *Phaseolus vulgaris* L. and non-inoculated controls (C) and irrigated with 50 µM folic acid (vitamin B9) or not (control, C). 35 days after mycorrhizal and bacterial mixed inoculation. Means ± standard deviation with n=4. Different letters above bars indicate significant differences (Tukey H $P \leq 0.05$).

CONCLUSIONS

The positive impact of vitamin B9 on *P. vulgaris* weight aligns with its established role in growth-promoting metabolic pathways. Additionally, this study found that vitamin B9 reduced proline in leaves. The enhanced leaf area with microbial inoculation emphasizes the contribution of symbiosis to an increased leaf greenness related to N content in leaf acquisition and photosynthetic efficiency. The lack of an additive effect of vitamin B9 on leaf area in inoculated *P. vulgaris* L. indicates a complex interplay among nutrient availability, symbiotic interactions, and *P. vulgaris* L. physiological responses. A biological and/or physical restriction might limit how much *P. vulgaris* L. can utilize vitamin B9, and it cannot be further enhanced by the inoculation with the microbes.

ETHICS STATEMENT

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF SUPPORTING DATA

All data generated or analysed during this study is included in this published article.

COMPETING INTERESTS

The authors declare no competing interests.

FINANCING

Not applicable.

AUTHORS' CONTRIBUTIONS

Conceptualization: J.B. Methodology: J.B., S.L.C.R., N.M.M., and E.M.R. Research: J.B., E.M.R., S.L.C.R., and N.M.M. Resources: E.M.R., N.M.M., and S.L.C.R. Monitoring: S.L.C.R., E.M.R., and N.M.M. Writing, preparing the original draft: J.B. Writing, review and editing: S.L.C.R., N.M.M., and E.M.R.

ACKNOWLEDGEMENTS

JBT thanks the División de Ciencias Biológicas y de la Salud of the Universidad Autónoma Metropolitana (UAM) and CONAHCyT for the scholarship (286223) to pursue a doctoral degree in the Program of Doctorado en Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana. JBT also thanks the Center of Genomic Sciences of Universidad Nacional Autónoma de México (UNAM) and Universidad Veracruzana (UV) for the resources provided to the development of this project.

REFERENCES

Al-Elwany, O. A. A. I., Hemida, K. A., Abdel-Razek, M. A., El-Mageed, T. A. A., El-Saadony, M. T., AbuQamar, S. F., … & Taha, R. S. (2022). Impact of folic acid in modulating antioxidant activity, osmoprotectants, anatomical responses, and photosynthetic efficiency of *Plectranthus amboinicus* under salinity conditions. *Frontiers in Plant Science*, *13,* 1-15. https://doi.org/10.3389/fpls.2022.887091

Aljuaid, B. S., Mukherjee, S., Sayed, A. N., El-Gabry, Y. A., Omar, M. M. A., Mahmoud, S. F., … & Hamada, M. M. A. (2022) Folic acid reinforces maize tolerance to sodic-alkaline stress through modulation of growth, biochemical and molecular mechanisms. *Life 12*(9),1-16. https://doi.org/10.3390/life12091327

Al-Said, M. A., & Kamal, A. M. (2008). Effect of foliar spray with folic acid and some amino acids on flowering yield and quality of sweet pepper. *Journal of Plant Production*, *33*(10), 7403–7412. https://doi.org/10.21608/jpp.2008.171240

Alsamadany, H., Mansour, H., Elkelish, A., & Ibrahim, M. F. M. (2022). Folic acid confers tolerance against salt stress-induced oxidative damages in snap beans through regulation growth, metabolites, antioxidant machinery and gene expression. *Plants*, *11*(11), 1-16. https://doi.org/10.3390/plants11111459

Augé, R. M., Stodola, A. J. W., Ebel, R. C., & Duan, X. (1995). Leaf elongation and water relations of mycorrhizal sorghum in response to partial soil drying: Two *Glomus* species at varying phosphorus fertilization. *Journal of Experimental Botany*, *46*(3), 297–307. https://doi.org/10.1093/jxb/46.3.297

Alvarez, M. E., Savouré, A., & Szabados, L. (2022). Proline metabolism as regulatory hub. *Trends in Plant Science*, *27*(1), 39-55.

Ayala-Rodríguez, J. Á., Barrera-Ortiz, S., Ruiz-Herrera, L. F., & López-Bucio, J. (2017). Folic acid orchestrates root development linking cell elongation with auxin response and acts independently of the target of rapamycin signaling in *Arabidopsis thaliana*. *Plant Science*, *264*, 168-178. https://doi.org/10.1016/j.plantsci.2017.09.011

Banuelos, J., Martínez-Romero, E., Montaño, N. M., & Camargo-Ricalde, S. L. (2021). Folates in legume root nodules. *Physiologia Plantarum*, *171*(3), 447-452. https://doi.org/10.1111/ppl.13218

Banuelos, J., Martínez-Romero, E., Montaño, N. M., & Camargo-Ricalde, S. L. (2023). *Rhizobium tropici* and riboflavin amendment condition arbuscular mycorrhiza colonization in *Phaseolus vulgaris* L. *Agronomy*, *13*(3), 1-13. https://doi.org/10.3390/agronomy13030876

Bañuelos, J., Trejo, D., Alarcon, A., Lara, L., Moreira, C., & Cruz, S. (2012). The reduction in proline buildup in mycorrhizal plants affected by nematodes. *Journal of Soil Science and Plant Nutrition*, *12*(2), 263-270. https://doi.org/10.4067/S0718-95162012000200006

Bar-Even, A., Noor, E., Flamholz, A., & Milo, R. (2013). Design and analysis of metabolic pathways supporting formatotrophic growth for electricity-dependent cultivation of microbes. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1827*(8-9), 1039-1047. https://doi.org/10.1016/j.bbabio.2012.10.013

Basset, G. J. C., Quinlivan, E. P., Gregory, J. F., & Hanson, A. D. (2005). Folate synthesis and metabolism in plants and prospects for biofortification. *Crop Science*, *45*(2), 449-453. https://doi.org/10.2135/cropsci2005.0449

Bekuzarova, S. A., Kozyrev, A. K., Shabanova, I. A., Lushenko, G. V., & Weissfeld, L. I. (2020). Enhancing of nitrogen fixation by legumes. *BIO Web of Conferences*, *23*, 1-6. https://doi.org/10.1051/bioconf/20202302006

Burguieres, E., McCue, P., Kwon, Y. I., & Shetty, K. (2007). Effect of vitamin C and folic acid on seed vigor response and phenolic-linked antioxidant activity. *Bioresource Technology*, *98*(7), 1393-1404. https://doi.org/10.1016/j.biortech.2006.05.046

Bates, L. S., Waldren, R. P. A., & Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil, 39*, 205-207. https://doi.org/10.1007/BF00018060

Cela, F., Avio, L., Giordani, T., Vangelisti, A., Cavallini, A., Turrini, A., … & Incrocci, L. (2022). Arbuscular mycorrhizal fungi increase nutritional quality of soilless grown lettuce while overcoming low phosphorus supply. *Foods*, *11*(22), 1-12. https://doi.org/10.3390/foods11223612

De Brujin, F. J. (2019). The common symbiotic signaling pathway (CSSP or SYM). In *The model legume Medicago truncatula*. (pp. 521-531). Nueva Jersey, USA: John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119409144.part8

Esfandiari, E., Enayati, W., Sabaghnia, N., & Janmohammadi, M. (2012). Effects of folic acid on seed germination properties and seedling growth of wheat. *Albanian Journal of Agricultural Sciences*, *11*(3), 185-192.

Farouk, S., & Abdul-Qados, A. M. S. (2018). Enhancing seed quality and productivity as well as physio-anatomical responses of pea plants by folic acid and/or hydrogen peroxide application. *Scientia Horticulturae*, *240*, 29-37. https://doi.org/10.1016/j.scienta.2018.05.049

Ghignone, S., Salvioli, A., Anca, I., Lumini, E., Ortu, G., Petiti, L., … & Bonfante, P. (2012). The genome of the obligate endobacterium of an AM fungus reveals an interphylum network of nutritional interactions. *The ISME Journal*, *6*(1), 136-145. https://doi.org/10.1038/ismej.2011.110

Giovannetti, M., & Mosse, B. (1980). An evaluation technique for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytologist*, *84*(3), 1-14. https://doi.org/10.1111/j.1469-8137.1980.tb04556.x

Gorelova, V., Ambach, L., Rébeillé, F., Stove, C., & Straeten, D. V. D. (2017). Folates in plants: research advances and progress in crop biofortification. *Frontiers in Chemistry*, *5*(21), 1-20. https://doi.org/10.3389/fchem.2017.00021

Hernández-Álvarez, C., Peimbert, M., Rodríguez-Martin, P., Trejo-Aguilar, D., Alcaraz, L. D. (2023). A study of microbial diversity in a biofertilizer consortium. *Plos One, 18*(8), 1-20. https://doi.org/10.1371/journal.pone.0286285

Hwang, S. Y., Kang, Y. J., Sung, B., Jang, J. Y., Hwang, N. L., Oh, H. J., ... & Kim, N. D. (2018). Folic acid is necessary for proliferation and differentiation of C2C12 myoblasts. *Journal of Cellular Physiology*, *233*(2), 736-747. https://doi.org/10.1002/jcp.25989

Ibrahim, M. F. M., Ibrahim, H. A., & El-Gawad, H. G. A. (2021). Folic acid as a protective agent in snap bean plants under water deficit conditions. *The Journal of Horticultural Science and Biotechnology*, *96*(1), 94-109. https://doi.org/10.1080/14620316.2020.1793691

Jabrin, S., Ravanel, S., Gambonnet, B., Douce, R., & Rébeillé, F. (2003). One-carbon metabolism in plants. Regulation of tetrahydrofolate synthesis during germination and seedling development. *Plant Physiology*, *131*(3), 1431-1439. https://doi.org/10.1104/pp.016915

Kaler, A. S., Abdel-Haleem, H., Fritschi, F. B., Gillman, J. D., Ray, J. D., Smith, J. R., & Purcell, L. C. (2020). Genome-wide association mapping of dark green color index using a diverse panel of soybean accessions. *Scientific Reports*, *10*(1), 1-11. https://doi.org/10.1038/s41598-020-62034-7

Karcher, D. E., & Richardson, M. D. (2003). Quantifying turfgrass color using digital image analysis. *Crop Science*, *43*(3), 943-951. https://doi.org/10.2135/cropsci2003.9430

Kavanova, M., Grimoldi, A. A., Lattanzi, F. A., & Schnyder, H. (2006). Phosphorus nutrition and mycorrhiza effects on grass leaf growth. P status- and size-mediated effects on growth zone kinematics. *Plant, Cell & Environment*, *29*(4), 511-520. https://doi.org/10.1111/j.1365-3040.2005.01428.x

Kayoumu, M., Iqbal, A., Muhammad, N., Li, X., Li, L., Wang, X., … & Dong, Q. (2023). Phosphorus availability affects the photosynthesis and antioxidant system of contrasting low-P-tolerant cotton genotypes. *Antioxidants*, *12*(2), 1-23. https://doi.org/10.3390/antiox12020466

Keshavarz, H., Moghadam, R.S.G. (2017). Seed priming with colbalamin (vitamin B12) provides significant protection agains salinity stress in the common bean. *Rhizosphere*. *3*(1)143-149. https://doi.org/10.1016/j.rhisph.2017.04.010

Khavazi, K., Rejali, F., Seguin, P., & Miransari, M. (2007). Effects of carrier, sterilization method, and incubation on survival of *Bradyrhizobium japonicum* in soybean (*Glycine max* L.) inoculants. *Enzyme and Microbial Technology*, *41*(6-7), 780-784. https://doi.org/10.1016/j.enzmictec.2007.06.011

Kim, S., Lindner, S. N., Aslan, S., Yishai, O., Wenk, S., Schann, K., & Bar-Even, A. (2020). Growth of *E. coli* on formate and methanol via the reductive glycine pathway. *Nature Chemical Biology*, *16*, 538-545. https://doi.org/10.1038/s41589-020-0473-5

Li, Y., Luo, J., Chen, R., Zhou, Y., Yu, H., Chu, Z., … & Ouyang, B. (2023). Folate shapes plant root architecture by affecting auxin distribution. *The Plant Journal*, *113*(5), 969-985. https://doi.org/10.1111/tpj.16093

Llerena-Ramos, L.T., Rodríguez-Rodríguez, S., Reyes-Pérez, J.J., López-Álvarez, S., Jiménez-Pizarro, M., & Espinosa-Palomeque, B. (2025). Microorganismos Benéficos y Compost Líquido Enriquecido con Silicio: Alternativas para la Producción Agroecológica del Cultivo de Arroz. *Terra Latinoamericana, 43*, 1-15. https://doi.org/10.28940/terra.v43i.2108

Ma, Y., Wei, Z., Liu, J., Liu, X., & Liu, F. (2021). Growth and physiological responses of cotton plants to salt stress. *Journal of Agronomy and Crop Science*, *207*(3), 565-576. https://doi.org/10.1111/jac.12484

Mashatleh, M., Assayed, A., Al-Hmoud, N., Ali, H. A., Al-Abaddi, R., & Alrwashdeh, M. (2024). Enhancing sustainable solutions for food security in Jordan: using bacterial biofertilizer to promote plant growth and crop yield. *Frontiers in Sustainable Food Systems, 8*, 1-10. http://doi.org/10.3389/fsufs.2024.1423224

Minitab (2017). *Minitab Statistical Software User´s Guide. Version 18.1.* State College, PA, USA: Minitab Inc.
Petiole LTD. (2019). *User´s guide Petiole Pro: Plant Phenotyping (Version 2.0.1).* London, United Kingdom: Petiole LTD.
Phillips, J. M., & Hayman, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society*, *55*, 158-161.
Piya, S., Pantalone, V., Zadegan, S. B., Shipp, S., Lakhssassi, N., Knizia, D., … & Hewezi, T. (2023). Soybean gene co-expression network analysis identifies two co-regulated gene modules associated with nodule formation and development. *Molecular Plant Pathology*, *24*(6), 628-636. https://doi.org/10.1111/mpp.13327
Pozo, M. J., López-Ráez, J. A., Azcón-Aguilar, C., & García-Garrido, J. M. (2015). Phytohormones as integrators of environmental signals in the regulation of mycorrhizal symbioses. *New Phytologist*, *205*(4), 1431-1436. https://doi.org/10.1111/nph.13252
Rashidi, S., Yousefi, A. R., Pouryousef, M., & Goicoechea, N. (2021). Mycorrhizal impact on competitive relationships and yield parameters in *Phaseolus vulgaris* L. weed mixtures. *Mycorrhiza*, *31*, 599-612. https://doi.org/10.1007/s00572-021-01046-1
Rhezali, A., Purcell, L. C., Roberts, T. L., & Greub, C. E. (2018). Predicting nitrogen requirements for maize with the Dark Green Color Index under experimental conditions. *Agronomy Journal*, *110*(3), 1173-1179. https://doi.org/10.2134/agronj2017.09.0543
Rorie, R. L., Purcell, L. C., Karcher, D. E., & King, C. A. (2011a). The Assessment of leaf nitrogen in corn from digital images. *Crop Science*, *51*(5), 2174-2180. https://doi.org/10.2135/cropsci2010.12.0699
Rorie, R. L., Purcell, L. C., Mozaffari, M., Karcher, D. E., King, C. A., Marsh, M. C., & Longer, D. E. (2011b). Association of "greenness" in corn with yield and leaf nitrogen concentration. *Agronomy Journal, 103*(2), 529-535. https://doi.org/10.2134/agronj2010.0296
Rosenblueth, M., & Martínez-Romero, E. (2004). *Rhizobium etli* maize populations and their competitiveness for root colonization. *Archives of Microbiology*, *181*, 337-344. https://doi.org/10.1007/s00203-004-0661-9
Seddas, P. M., Arias, C. M., Arnould, C., Van Tuinen, D., Godfroy, O., Benhassou, H. A., ... & Gianinazzi-Pearson, V. (2009). Symbiosis-related plant genes modulate molecular responses in an arbuscular mycorrhizal fungus during early root interactions. *Molecular Plant-Microbe Interactions*, *22*(3), 341-351. https://doi.org/10.1094/MPMI-22-3-0341
Sheteiwy, M. S., El-Sawah, A. M., Korany, S. M., Alsherif, E. A., Mowafy, A. M., Chen, J., … & AbdElgawad, H. (2022). Arbuscular mycorrhizal fungus *Rhizophagus irregularis* impacts on physiological and biochemical responses of ryegrass and chickpea plants under beryllium stress. *Environmental Pollution*, *315*, 120356. https://doi.org/10.1016/j.envpol.2022.120356
Tao, J., Dong, F., Wang, Y., Chen, H., & Tang, M. (2022). Arbuscular mycorrhizal fungi enhance photosynthesis and drought tolerance by regulating MAPK genes expressions of *Populus simonii* × *P. nigra*. *Physiologia Plantarum*, *174*(6), e13829. https://doi.org/10.1111/ppl.13829
Van-Wilder, V., De-Brouwer, V., Loizeau, K., Gambonnet, B., Albrieux, C., Straeten, D. V. D., … & Ravanel, S. (2009). C1 metabolism and chlorophyll synthesis: The Mg-protoporphyrin IX methyltransferase activity is dependent on the folate status. *New Phytologist*, *182*(1), 137-145. https://doi.org/10.1111/j.1469-8137.2008.02707.x
Voysest, O. (1983). *Variedades de frijol en América Latina y su origen*. Cali, Colombia: Centro International de Agricultura Tropical (CIAT).
Wang, K. L. C., Li, H., & Ecker, J. R. (2002). Ethylene biosynthesis and signaling networks. *The Plant Cell*, *14*(1), 131-151. https://doi.org/10.1105/tpc.001768
Wang, N., Fu, F., Wang, H., Wang, P., He, S., Shao, H., … & Zhang, X. (2021). Effects of irrigation and nitrogen on chlorophyll content, dry matter and nitrogen accumulation in sugar beet (*Beta vulgaris* L.). *Scientific Reports*, *11*(1), 1-9. https://doi.org/10.1038/s41598-021-95792-z
Yang, G., Bhuvaneswari, T. V., Joseph, C. M., King, M. D., & Phillips, D. A. (2002). Roles for Riboflavin in the *Sinorhizobium* -Alfalfa Association. *Molecular Plant-Microbe Interactions, 15*(5), 1-7. https://doi.org/10.1094/MPMI.2002.15.5.456
Ye, Q., Wang, H., & Li, H. (2022). Arbuscular mycorrhizal fungi improve growth, photosynthetic activity, and chlorophyll fluorescence of *Vitis vinifera* L. cv. Ecolly under drought stress. *Agronomy*, *12*(7), 1-14. https://doi.org/10.3390/agronomy12071563
Zrenner, R., Stitt, M., Sonnewald, U., & Boldt, R. (2006). Pyrimidine and purine biosynthesis and degradation in plants. *Annual Review of Plant Biology*, *57*, 805-836. https://doi.org/10.1146/annurev.arplant.57.032905.105421