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Rizobacterias Promotoras del Crecimiento Vegetal (PGPR) y su aporte en la nutrición mineral de tomate (*Lycopersicon sculentum* L.)

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RESUMEN

El nitrógeno (N), el fósforo (P) y el potasio (K) son macronutrientes esenciales para el crecimiento y productividad de las plantas. Sin embargo, no siempre están disponibles para las plantas en el suelo. Las rizobacterias promotoras del crecimiento vegetal (PGPR) son bacterias del suelo que pueden colonizar la rizósfera y mejorar la disponibilidad de estos nutrientes a través de los mecanismos de fijación biológica de nitrógeno y solubilización de P y K. El objetivo de esta investigación fue seleccionar rizobacterias promotoras del crecimiento vegetal nativas, que fijen N_2 y solubilicen P y K de minerales del suelo, para su uso como biofertilizantes en el cultivo de tomate. La investigación se desarrolló en tres etapas. En la primera etapa, se estudiaron 72 rizobacterias de la Colección de Microorganismos de la Facultad de Agronomía. Se evaluó y cuantificó la capacidad de las rizobacterias para fijar N_2 y solubilizar P y K *in vitro,* en los medios de cultivo Burk's libre de N, Pikovskaya y Aleksandrov, respectivamente. Se clasificaron 15 rizobacterias por su capacidad de crecimiento en el medio libre de N y por formar halos de solubilización de P y de K. De estas 15 rizobacterias se seleccionaron 4 por su capacidad de fijar N_2 (0.6 a 1.7 mg L^{-1} NH_4^+), 3 por solubilizar P (144.4 a 308.6 mg L^{-1} $P\text{-}PO_4$) y 5 por solubilizar K (25.0 a 37.0 mg L^{-1} K) (correlación cofenética > 0.8). Estas rizobacterias fueron aisladas de los cultivos de tomate, lenteja, ají, haba y lechuga, cultivados en suelos Andisoles y Alfisoles. En base en la secuenciación del gen 16SrRNA, estas rizobacterias se identificaron como *Pseudomonas gessardi, P. koreensis, P. brassicacearum*, *P. marginalis*, *Acinetobacter calcoaceticus y Rahnella aquatica*. En la segunda etapa, se evaluó la capacidad endofítica y el efecto de la inoculación de las rizobacterias seleccionadas *in vitro* en la disponibilidad de NPK y la promoción del crecimiento en plántulas de tomate 'Cal Ace' cultivadas en suelo con baja fertilidad: 7.0 mg kg^{-1} N disponible, 19.20 mg kg^{-1} P disponible, 40.90 mg kg^{-1} K disponible. Se realizaron tres experimentos, uno para cada tipo de bacteria seleccionada. Las bacterias solubilizadoras de fósforo no mostraron un efecto positivo en el crecimiento de las plantas o un aumento en el suelo disponible P. La bacteria fijadora de N_2, Tmt-16 aumentó el crecimiento de las raíces en 23.57 %, generó el mayor contenido de N en el tejido vegetal, 2.60 % y en el suelo produjo la mayor cantidad de amonio,1.95 mg kg_{-1} y N disponible, 2.95 mg kg^{-1}. Las bacterias

solubilizadoras de K; Ls-C21, Ltj-62 y LsC-58 generaron de 17.0 a 19.0 mg kg^{-1} de K disponible y de 0.04 a 0.05 mg kg^{-1} de K intercambiable ($p \leq 0.05$). Con base en la huella genética, ninguna de las rizobacterias tuvo la capacidad de establecerse como endófita. En la tercera etapa, se estudió el efecto de la interacción entre las rizobacterias seleccionadas *in vitro* y distintos niveles de fertilización en la biodisponibilidad de NPK y la promoción de crecimiento de plantas de tomate. Se realizaron tres experimentos: (1) Disponibilidad de N_2, se evaluaron; 3 rizobacterias + 1 Control, en 3 niveles de fertilización-N; 0, 50 y 100 % (%N); (2) Solubilización de P, se estudiaron 3 rizobacterias + 1 Control, en 2 niveles de fertilización-P, 50 y 100% (%P), y (3) Solubilización de K, se estudiaron 5 rizobacterias + 1 Control, en 3 niveles de fertilización-K; 0, 50 y 100 % (%K). En el experimento de disponibilidad de N, la aplicación de Tmt-16+100%N incrementó 51.6 y 43.8 % la concentración de NO_3^- y N-disponible en el suelo, las aplicaciones de Hb-142+50%N y Tmt-16+0%N incrementaron el contenido de N en el tejido vegetal en un 19.8 y 71.2 %, y en la promoción de crecimiento, Hb-142+50%N y Tmt1-107+50%N, generaron un 29.2 y 24.6 % más de materia seca. En el experimento de solubilización de P, los tratamientos no mostraron un efecto positivo en la biodisponibilidad de P ni en la promoción de crecimiento. En el experimento de solubilización de K, los tratamientos; Ltj-62+100%K, LsC-54+50%K y LsC-21+0%K incrementaron de 14 a 60 % los valores de K intercambiable y disponible en el suelo. En la planta, los tratamientos no indujeron mayor contenido de K en el tejido vegetal y no promovieron el crecimiento. Los diversos efectos de la interacción entre las bacterias fijadoras de N_2, solubilizadoras de P y solubilizadoras de K con los distintos niveles de fertilización, brindan una amplia posibilidad del uso de estas rizobacterias en suelos con distintos niveles de fertilidad. Permitiendo generar una alternativa sostenible para la producción del cultivo de tomate. Además, estas rizobacterias fueron compatibles entre sí por lo que en un futuro pueden evaluarse en consorcio y contribuir a aumentar la eficiencia en el uso de nutrientes y la productividad en diversos cultivos para una producción agrícola sostenible.

Palabras clave: *Acinetobacter*, Feldespato-K, Fijación de N_2, solubilización de fosforo y potasio, *Pseudomonas*, *Rahnella*.

ABSTRACT

Nitrogen (N), phosphorus (P) and potassium (K) are essential macronutrients for plant growth and productivity. However, they are not always available for plants in the soil. Plant growth promoting rhizobacteria (PGPR) are soil bacteria that can colonize the rhizosphere and improve the availability of these nutrients through the mechanisms of biological nitrogen fixation and solubilization of P and K. The objective of this research was to select native plant growth promoting rhizobacteria, which fix N_2 and solubilize phosphorus or potassium from soil minerals, for use as biofertilizers in tomato. The research was carried out in three stages. In the first stage, 72 rhizobacteria from the Microorganism Collection of the Faculty of Agronomy were studied. The ability of rhizobacteria to fix N_2 and solubilize P and K *in vitro* was evaluated and quantified in Burk's N free media, Pikovskaya and Aleksadrov, respectively. Fifteen rhizobacteria were classified for their growth capacity in the N-free medium and for forming solubilization halos for P and K. Of these 15 rhizobacteria 4 were selected for their ability to fix N_2 (0.6 to 1.7 mg L-1 NH4 +), 3 for solubilizing P (144.4 to 308.6 mg L-1 P-PO4) and 5 for solubilizing K (25.0 to 37.0 mg L-1 K) (cofénetica correlation> 0.8). These rhizobacteria were isolated from tomato, lentil, chili, bean and lettuce crops, grown in Andisoles and Alfisols soils. Based on the sequencing of the 16SrRNA gene, these rhizobacteria were identified as *Pseudomonas gessardi, P. koreensis, P. brassicacearum, P. marginalis, Acinetobacter calcoaceticus and Rahnella aquatica.* In the second stage, the endophytic capacity and the effect of inoculation of the selected rhizobacteria *in vitro* on NPK availability and in growth promotion in 'Cal Ace' tomato seedlings grown in soil with low fertility (7.0 mg kg^{-1} N available, 19.20 mg kg^{-1} P available, 40.90 mg kg^{-1} K available) were evaluated. Three experiments were carried out, one for each type of bacteria selected. Phosphorus solubilizing bacteria did not show a positive effect on plant growth or an increase in available soil P. The N_2 fixing bacteria, Tmt-16 increased root growth by 23.57%, generated the highest N content in the plant tissue, 2.60% and produced the highest amount of ammonium in the soil, 1.95 mg kg-1 and available N, 2.95 mg kg-1. K solubilizing bacteria; Ls-C21, Ltj-62 and LsC-58 generated 17.0 to 19.0 mg kg-1 of available K and 0.04 to 0.05 mg kg^{-1} of exchangeable K ($p \leq 0.05$). Based on the genetic footprint, none of the rhizobacteria

had the ability to establish themselves as endophytes in tomato. In the third stage, the effect of the interaction between the selected rhizobacteria *in vitro* and different levels of fertilization on the bioavailability of NPK and plant growth promotion were studied. Three experiments were performed: (1) Availability of N_2, were evaluated; 3 rhizobacteria + 1 Control, in 3 levels of fertilization-N; 0, 50 and 100% (% N); (2) Solubilization of P, 3 rhizobacteria + 1 Control were studied, in 2 fertilization levels-P, 50 and 100% (% P), and (3) Solubilization of K, 5 rhizobacteria + 1 Control were studied, in 3 K-fertilization levels; 0, 50 and 100% (% K). In the N availability experiment, the application of Tmt-16+100% N increased the concentration of NO_3^- and N-available in the soil 51.6 and 43.8 %, the applications of Hb-142+50% N and Tmt-16+0% N increased the content of N in plant tissue by 19.8 and 71.2 %, and in the growth promotion, Hb-142+50%N and Tmt1-107+50%N, generated 29.2 and 24.6 % more of dry matter. In the P solubilization experiment, the treatments did not show a positive effect on the bioavailability of P nor growth promotion effects. In the experiment of K solubilization, the treatments; Ltj-62+100%K, LsC-54+50%K and LsC-21+0%K increased the values of K-interchangeable and K-available in the soil from 14 to 60%. In the plant, the treatments did not induce a higher K content in the plant tissue and did not promote growth. The various effects of the interaction between N_2 fixing batteries, P solubilizers and K solubilizers with different levels of fertilization, provide a wide possibility of using these rhizobacteria in soils with different fertility levels. Allowing to generate a sustainable alternative for tomato crop production. In addition, these rhizobacteria were compatible with each other so that in the future they can be evaluated in consortium and contribute to increasing the efficiency in the use of nutrients and productivity in various crops for sustainable agricultural production.

Keywords: *Acinetobacter*, Feldspar-K, N_2 fixation, phosphorus and potassium solubilization, *Pseudomonas, Rahnella.*

I. CAPITULO 1: INTRODUCCIÓN GENERAL

El nitrógeno (N), el fósforo (P) y el potasio (K) son macronutrientes esenciales para el crecimiento y productividad de las plantas. Sin embargo, no siempre están disponibles para las plantas en el suelo. Las rizobacterias promotoras del crecimiento vegetal (PGPR) son bacterias del suelo que pueden colonizar la rizósfera y mejorar la disponibilidad de estos nutrientes a través de los mecanismos de fijación biológica de nitrógeno, reducción de N_2 a NH_3^- por el complejo enzimático nitrogenasa, y de la solubilización de P y K mediante la liberación de ácidos orgánicos e inorgánicos a través de acidólisis, quelación y complexólisis. Las PGPR pueden clasificarse como biofertilizantes cuando actúan como fuente de nutrición vegetal y fuente de enriquecimiento para reponer o reconstruir el ciclo de nutrientes entre el suelo, las raíces de las plantas y los microorganismos presentes. El mercado de biofertilizantes se valoró en 946,6 millones USD en 2015, con una proyección de la tasa compuesta anual del 14.08 %. Los biofertilizantes están cada vez más disponibles en el mercado de insumos agrícolas en Chile. Sin embargo, el 80 % corresponden a insumos importados. Los cuales no necesariamente han sido evaluados en los cultivos y las condiciones climáticas en las que se están aplicando. Por lo tanto, existe la necesidad de desarrollar tecnologías específicas para cada cultivo y región de Chile.

1.1. Importancia del suelo en la agricultura para la producción de alimentos

De acuerdo con la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO) “la seguridad alimentaria se consigue cuando las personas tienen, en todo momento, acceso físico y económico a alimentos seguros y nutritivos, en cantidad suficiente para satisfacer sus necesidades alimenticias” (FAO, 2009). Según estimaciones del Departamento de Asuntos Económicos y Sociales de la ONU, la población mundial en 2030 será de 8.5 mil millones, 9.7 mil millones en 2050 y 11.2 mil millones en el 2100 (ONU, 2015), por lo que las exigencias impuestas a la cadena agroalimentaria para suministrar alimentos en el futuro será uno de los mayores desafíos que enfrente la población humana (McAfee 2008).

La agricultura, la seguridad de la producción de alimentos y la preservación de los recursos naturales están estrechamente vinculadas. Por lo que las investigaciones científicas deben estar dirigidas a contribuir en garantizar la eficiencia económica, la equidad social y la sostenibilidad ambiental. Este último se centra en la mitigación y adaptación al cambio climático, la biodiversidad y la preservación del agua y del suelo (Passeri et al., 2016). Todo tipo de vida terrestre depende de la calidad del suelo para su supervivencia. Por ende, la protección de este recurso natural debe ser una política nacional e internacional (Bautista-Cruz et al., 2004).

Un suelo de calidad debe tener la capacidad para funcionar dentro de los límites de un ecosistema natural o manejado, sostener la productividad de plantas y animales, mantener o mejorar la calidad del aire y del agua, y sostener la salud humana y el hábitat (Karlen et al., 1997). En la agricultura “el suelo” es un factor importante y clave para cumplir con las exigencias para producir y suministrar alimentos (FAO, 2015), por lo que la FAO designó al año 2015, como “El Año Internacional de los Suelos” con el objetivo de mostrar la necesidad de gestionar de forma sostenible este importante recurso (FAO, 2015). Esto debido a que existe un agotamiento de nutrientes en los suelos por la continua falta de reabastecimiento de nutrientes, afectando negativamente su calidad y la reducción de los rendimientos de los cultivos, por lo que este problema es una amenaza potencial para la seguridad alimentaria global y la sostenibilidad agrícola (Tan et al., 2005; Sheldrick et al., 2002).

1.2. Disponibilidad de nutrientes (NPK) en el suelo para la producción de alimentos

1.2.1. El suelo como fuente de nutrientes

El suelo contiene varios minerales que presentan elementos esenciales para la nutrición mineral de las plantas como el nitrógeno (N), fósforo (P) y potasio(K) (McAfee 2008). El NPK son requeridos en las funciones bioquímicas en la planta (fotosíntesis y respiración), al formar parte de compuestos orgánicos y constituir diversos aminoácidos, proteínas y ácidos nucleicos, nucleótidos, coenzimas, hexoaminas, entre otros (Dobbelaere et al., 2003; Patiño y Sánchez, 2012; Salisbury and Ross, 1992).

Nitrógeno. De todos los nutrientes del suelo necesarios para el crecimiento de las plantas, el nitrógeno es el elemento más limitante (Vitousek et al., 1997), debido a que el mayor reservorio se encuentra en la atmósfera (99 % N_2) y no en el suelo, por lo que los sistemas productivos agrícolas estan limitados por el suministro del N biológicamente disponible; fijación biológica por microorganismos y sales aportadas por las lluvias (Robertson y Groffman, 2013).

Los diferentes estados oxidativos del N en su ciclo ocurren mediante los procesos biológicos de mineralización, inmovilización, nitrificación y desnitrificación, (Robertson y Groffman, 2013). Las reservas de N están constituidas por la materia orgánica (MO) de descomposición rápida en medios biológicamente activos, quedando disponible para las plantas a través del proceso de mineralización (conversión de N orgánico a inorgánico), en el cual participan activamente los microorganismos.

En suelos agrícolas con poca cantidad de MO, la mineralización no proporciona a los cultivos cantidades suficientes de N inorgánico (Chotte et al., 2002), de ahí la necesidad de aportes de N inorgánico mediante fertilización química o aplicación de abonos orgánicos al suelo. Así mismo, el nitrógeno orgánico puede no estar disponible para las plantas en moléculas complejas o perderse por desnitrificación, erosión del suelo, lixiviado, volatilización y extracción por cosechas (Philippot y Germon, 2005).

Fósforo. En promedio, el suelo contiene 0.02 a 0.5 % del P total (Fernández *et al.,* 2007). Este nutriente mineral se añade al suelo en forma de fertilizantes, del cual sólo el 1 % es utilizado por las plantas y el resto se transforma rápidamente en complejos insolubles, precipitándose aproximadamente un 80 % de la adición de los fertilizantes fosfatados. Los aniones fosfato ($H_2PO_4^-$, HPO_4^{-2}) son extremadamente reactivos y forman complejos metálicos con el Ca en suelos calcáreos y con el Fe^{3+} y Al^{3+} en suelos ácidos (Hariprasad y Niranjana, 2009; Jorquera et al., 2008; Qureshi et al., 2012), los cuales ocupan 3.95 billones de hectáreas, correspondientes al 30 % de la superficie terrestre (Fageria y Baligar, 2008). En general, estos suelos son deficientes en fósforo (P) disponible para los cultivos, debido a su elevada capacidad de fijación de P, esto conlleva a una deficiente nutrición mineral de las plantas y como

consecuencia, existe la necesidad de una aplicación frecuente de fertilizantes P, lo cual es costoso y e indeseable para el medio ambiente (Patiño y Sánchez, 2012).

Aunado a esto, la explotación de reservas globales de la roca fosfórica para su uso como fertilizantes, ha tenido impactos ambientales negativos debido al agotamiento inminente de las reservas globales de la roca fosfórica (Cordell et al., 2009), razones por las que se deben de generar alternativas sostenibles que permitan satisfacer los requerimientos de P de los cultivos en la agricultura moderna (Patiño y Sánchez, 2012).

Potasio. El K es uno de los elementos más abundantes en el suelo, y uno de los siete elementos más comunes en la corteza terrestre (Syers 2003). El contenido de potasio promedio total de los suelos es de 1.52 % (Mengel y Kirkby 2001). Sin embargo, el total de K está bastante mal correlacionada con el K disponible y rara vez se utiliza al K para describir el estado de fertilidad del suelo. La fuente inmediata de K para las plantas es la pequeña cantidad que está presente en la solución del suelo, la cual varía su concentración media del 1 a 2 % (Goldstein, 1994). Sin embargo, el agotamiento del potasio disponible en el suelo se ha reducido debido a la eliminación de cultivos, la lixiviación, escorrentía y la erosión de los suelos (Xie, 1998).

1.2.2. Agotamiento de nutrientes en el suelo y la reducción en la producción

La intensificación del uso de la tierra para la producción agrícola y la aplicación inadecuada de insumos externos ha provocado el agotamiento de los nutrientes del suelo, provocando a largo plazo la disminución de los rendimientos de los cultivos en muchas zonas de África, Asia y América Latina, poniendo en riesgo la seguridad alimentaria de países en desarrollo.

En un estudio a nivel mundial sobre el agotamiento de nutrientes del suelo y la reducción de rendimiento en alimentos básicos realizado por Tan et al (2005), se reportó que no existe un país sin problemas de nutrientes en sus sistemas de producción. El déficit total de NPK en la superficie global cultivada con trigo (*Triticum aestivum* L.), arroz (*Oryza sativa*), maíz (*Zea mays* L.) y cebada (*Hordeum vulgare*) en el año 2000, fue afectada en un 56 % por déficit de N a una tasa promedio de 17.4 kg ha^{-1} $año^{-1}$, en un 80 % por déficit de P a 5.0 kg ha^{-1} $año^{-1}$ y en un 56 % por déficit de K

a una tasa promedio de 38.7 kg ha^{-1} año $^{-1}$, por lo que el K fue el nutriente que presentó mayor déficit, 60 %, el N 28 % y el P 12 %.

El déficit de nutrientes en el suelo afectó la pérdida global de producción total en un 41 % para el arroz, 33 % en el trigo, 24 % en el maíz y 3 % en la cebada. Estas pérdidas de producción global total se produjeron en un 80 % en países en desarrollo y menos adelantados y un 20 % en países desarrollados. El área afectada en los países en desarrollo fue de 175 Mha (57 % de área cultivada) para N, 266 Mha (86 %) en P y 283 Mha (91 %) para K; en los países menos desarrollados, 31 Mha (69 %) para N, 32 Mha 70 %) para P y 31 Mha (69 %) para K y en los países desarrollados, 108 Mha (52 %) para N y 151 Mha (73 %) en P.

1.3. Suministro de nutrientes al suelo para la producción de alimentos

1.3.1. Impacto de fertilización química

Como solución inmediata al problema de agotamiento de nutrientes en el suelo y cumplir con las exigencias impuestas a la agricultura para suministrar alimento, se han utilizado gran cantidad de fertilizantes químicos para que de esta manera aumenten los rendimientos de los cultivos (Salgado-García y Núñez-Escobar, 2010; Ahlgren et al., 2008; Hartz, 2005).

A nivel mundial, en 2018 la utilización de fertilizantes sobrepasará los 200 millones de toneladas, con un aumento de 1,4 % en fertilizantes nitrogenados, 2.2 % en fosfatos y un 2.6 % en potásicos. En América Latina y el Caribe el uso de los fertilizantes crecerá un 3.3 % anual y dependerán de importaciones (FAO, 2015).

Los fertilizantes químicos son obtenidos a partir del petróleo, por lo que su precio está sujeto a la cotización internacional de este combustible fósil, por ende, el uso de fertilizantes químicos puede incrementar los costos de producción hasta en 60 % en algunos cultivos (Aguado- Santacruz et al., 2012), esto ha llevado a un aumento en el costo de la producción de alimentos y en la disminución de los ingresos del productor (Menna et al., 2014). El alto valor de los cultivos (alimentos) generalmente ocasiona que los agricultores apliquen cantidades excesivas de fertilizantes químicos (Hartz, 2005) para asegurar un rendimiento adecuado que les permita tener ingresos

económicos. El mal manejo de estos insumos es un tema sensible ya que ha generado impactos ambientales negativos (Camelo et al., 2011), principalmente con la fertilización nitrogenada.

1.3.1.1. Suministro de N y contaminación ambiental

El uso de N reactivo para la producción de alimentos ha tenido efectos secundarios sobre el ciclo N, la intensificación de la agricultura; su expansión en bosques y pastizales ha liberado aproximadamente 40 Tg año^{-1} (Vitousek et al., 1997). Durante los últimos 100 años, el aporte anual de N reactivo creado antropógenicamente ha aumentado dramáticamente debido a: 1) aumentos moderados en el uso de leguminosas en la agricultura (15 a 30 Tg N año^{-1}), 2) grandes aumentos en la combustión de combustibles fósiles (1 Tg N año^{-1} en 1860 vs 25 Tg N año^{-1} en 2000) y 3) aumentos en el uso del fertilizante N derivado del proceso de Haber-Bosch para la producción de alimentos (cero, previo al siglo 20, actualmente 110 Tg N año^{-1}). La entrada de N reactivo ha aumentado más de 10 veces en 100 años, y alrededor del 85 % ha sido utilizada por la agricultura para apoyar a la población humana cada vez mayor durante el mismo período (Robertson y Groffman, 2013). El uso excesivo de fertilizantes nitrogenados como urea ha generado lixiviación, eutrofización de ambientes terrestres y acuáticos y pérdida del ozono estratosférico (Gruber y Galloway, 2008).

1.3.2. Uso de microorganismos en el ciclaje de nutrientes para la producción agrícola

El suelo es un ecosistema natural en el cual proliferan numerosos microorganismos diferentes (Cassán et al., 2009). La posibilidad de utilizar microorganismos del suelo que favorecen la nutrición y desarrollo de las plantas ofrece nuevas alternativas para incrementar el rendimiento y mejorar el uso de los fertilizantes minerales (Fuentes-Ramirez y Caballero-Mellado, 2005).

Como una alternativa de solución a los problemas de agotamiento de nutrientes en el suelo, su contaminación por exceso de fertilización y la necesidad de suplir la demanda de fertilizantes químicos para la producción de alimentos, la FAO dentro del

primer principio de “Construir una visión común para la alimentación y la agricultura sostenible”, plantea como alternativa el “uso de microorganismos en el ciclaje de los nutrientes” (FAO, 2015).

Las bacterias forman parte de los microorganismos del suelo, por lo menos existen 104 taxones bacterianos y 10^{10}-10^{11} diferentes números de bacterias por gramo de suelo (Torsvik et al., 1990). Las bacterias que habitan en la rizósfera o zona del suelo influenciada por las raíces son llamadas Rizobacterias (Cassán et al., 2009). La rizósfera es un sistema dinámico donde se detecta la máxima actividad microbiana, en el cual las interacciones y la comunicación entre raíz y microorganismo juegan un papel muy importante en el mantenimiento del crecimiento y productividad vegetal (Hayat et al., 2010; Curl y Truelove, 1986). Estos microorganismos son capaces de asimilar formas no disponibles para la planta y transformarlas, hasta la obtención de formas asimilables para las células vegetales (Camelo et al., 2011). Kloepper y Schroth en 1978 introdujeron el término "rizobacterias promotoras del crecimiento de las plantas (PGPR: del inglés *Plant growth promoting rhizobacteria*)" para estos microorganismos beneficiosos, allanando el camino para mayores descubrimientos.

1.4. Uso de las rizobacterias promotoras del crecimiento vegetal (PGPR) en la nutrición mineral de cultivos

Las rizobacterias promotoras del crecimiento vegetal o PGPR por sus siglas en inglés, Plant Growth Promoting Rhizobacteria (Davison, 1988), son bacterias vivas aisladas principalmente de la rizósfera (Bashan et al., 2014) que promueven el crecimiento de las plantas mediante una amplia variedad de mecanismos (Pii et al., 2015; Davison,1988).

Varios estudios ponen de relieve que la inoculación de plantas con PGPR puede tener efectos considerables en la planta tanto a nivel fisiológico como molecular, lo que sugiere la posibilidad de que la biota del suelo pueda estimular a las plantas a ser más eficientes en la recuperación de nutrientes del suelo (Pii et al., 2015).

Las PGPR incrementan la disponibilidad de nutrimentos en la rizósfera al influir en el metabolismo de las plantas y mejorar su nutrición mediante mecanismos directos

como: fijación de nitrógeno; síntesis de fitohormonas (auxinas, giberelinas, citocininas), vitaminas y enzimas; solubilización de P inorgánico y mineralización de P orgánico; oxidación de sulfuros; incremento en la permeabilidad de la raíz; producción de nitritos; acumulación de nitratos; reducción de la toxicidad por metales pesados y de la actividad de la enzima ACC desaminasa (Glick 1995; Dobbelaere et al., 2003) y solubilización de K de formas no disponible de minerales que contienen K (Malinovskaya et al., 1990; Sheng y Huang, 2002; Hu et al., 2006; Zhang and Kong 2014; Meena et al., 2015a; Meena et al., 2015b).

1.4.1. Fijación de nitrógeno atmosférico

Mecanismo de acción. Para la fijación biológica de N, las bacterias utilizan como energía las fuentes de carbono proporcionadas por las plantas vía la fotosíntesis (Mantilla-Paredes et al., 2009) para luego convertir el N_2 en amoniaco a través de la actividad del complejo enzimático llamado nitrogenasa que está constituido por dos metaloproteínas: la proteína (I), llamada hierro-molibdeno-proteína, y la proteína (II), llamada hierro-proteína (Baca et al., 2000); la enzima requiere de la colaboración de otras dos proteínas: ferrodoxina y avodoxina, que actúan como donadores de electrones y reductores naturales de la nitrogenasa. Los electrones son transportados a la nitrogenasa por la ferrodoxina y llegan a la hierro-proteína, ésta activa a la Mo-Fe-proteína y se produce la reducción de N_2, siendo luego fijado como compuesto aminado (Hoffman et al., 2014; Carvalho et al., 2014).

La fijación biológica de N_2 reduce el problema de perdida de nitrógeno en comparación con los fertilizantes de nitrógeno reactivo, ya que al producirse dentro de organismos vivos el N fijado es asimilado rápidamente en constituyentes celulares (Robertson and Groffman, 2013). Poniendo en ventaja el uso de fijadores de N_2 en biofertilizantes comparado con los fertilizantes de N reactivo.

Géneros y/o especies estudiados. Se conoce un gran número de bacterias de vida libre o asociativas que fijan N_2, algunas de las que destacan por su potencial como biofertilizantes o como promotoras del crecimiento. Los géneros más conocidos son *Azospirillum, Azotobacter, Beijerinckia, Derxia, Pseudomonas* (Díaz *et al.,* 2001). Las estimaciones de fijación biológica de N_2 por bacterias de vida libre en ambientes

enriquecidos en suelo y en residuos vegetales oscilan generalmente entre 1 y 10 kg N ha^{-1} $año^{-1}$, en sistemas naturales el rango es inferior a estas estimaciones (Robertson y Groffman, 2013).

En el caso de los microorganismos simbióticos los más estudiados son los rizobios (Chebotar et al., 2001), bacterias que en simbiosis con leguminosas pueden llegar a fijar hasta 200 kg N ha^{-1} al año (Supanjani et al., 2006).

1.4.2. Solubilización de fósforo

Mecanismo de acción. La solubilización de P mineral es un proceso relacionado fundamentalmente con la producción y liberación de ácidos orgánicos por algunos microorganismos del suelo. Distintos ácidos orgánicos tales como: el ácido oxálico, el malónico, el succínico y el glucónico han sido identificados y relacionados con la solubilización de P mineral. La liberación de estos ácidos a la rizósfera provoca su acidificación y esto puede directamente incrementar la solubilización del fósforo (Hariprasad y Niranjana, 2009).

A diferencia de la solubilización de P mineral, la solubilización de P orgánico, es un proceso dirigido por enzimas, entre ellas tenemos: fosfatasas, que participan en la desfosforilación de los grupos fosfoester unidos a la materia orgánica; fitasas, que catalizan el proceso de hidrólisis del ácido fítico liberando de forma secuencial hasta seis grupos ortofosfatos libres y; las fosfonatasas y C-P liasas, enzimas que participan en la ruptura del enlace carbono-fósforo de los organofosfonatos. El principal trabajo les corresponde a las fosfatasas ácidas y a las fitasas, debido a la presencia predominante en sus sustratos y en el suelo (Pérez et al., 2007; Jha et al., 2009).

Géneros y/o especies estudiados. Los géneros bacterianos capaces de solubilizar fosfato son *Pseudomonas, Mycobacterium, Micrococus, Bacillus y Flavobacterium* (Díaz et al., 2001).

Ejemplos claros de bacterias fijadoras de N_2 y bacterias solubilizadoras de P, utilizadas ampliamente como principios activos de biofertilizantes en la agricultura moderna son *Azospirillum, Herbaspirillum, Burkholderia, Gluconacetobacter, Derxia,*

Beijerinckia y *Azotobacter* (Garrido et al., 2010)*, Herbaspirillum, Enterobacter, Bacillus, Alcaligenes, Klebsiella, Azotobacter y Pseudomonas* (Carvalho et al., 2014).

1.4.3. Solubilización de potasio desde minerales

Mecanismo de acción. Las rizobacterias solubilizadoras de potasio, contribuyen significativamente en la solubilización de K fijado en minerales del suelo hacia la solución del suelo (Menna et al., 2015). Sheng y Huang (2002) encontraron que la liberación de K de los minerales se ve afectada por el pH, oxígeno, y las cepas bacterianas utilizadas. Otros estudios mostraron que la solubilización del K por diferentes microorganismos varía con la naturaleza de los minerales (mica, muscovita, biotita feldespato) que contienen K y de las condiciones aerobias (Uroz et al., 2009; Lian, 1998; Lian et al., 2008; Chen et al., 2008 y Bin et al., 2010). Los mecanismos para la solubilización de K son: (i) la disminución del pH, o (ii) mediante la mejora de la quelación de los cationes unidos a K, y (iii) acidólisis de la zona circundante de microorganismo. La disminución en el pH del medio sugiere la liberación de ácidos orgánicos y protones por los microorganismos solubilizadores de K (Zarjani et al., 2013, Parmar y Sindhu, 2013 y Uroz et al., 2009). Esta acidólisis por los ácidos orgánicos producidos por los microorganismos rizosféricos cualquiera puede disolver directamente el K de los minerales como resultado de liberaciones lentas de K intercambiable (Römheld y Kirkby 2010). Por lo tanto, la síntesis y secreción de ácidos orgánicos por los microorganismos en el medio ambiente circundante acidifican las células del microorganismo y su entorno circundante que en última instancia conducen a la liberación de iones K del mineral-K por protonación y acidificación (Goldstein 1994). Los diversos tipos de ácidos orgánicos producidos por los microorganismos solubilizadores de K difieren entre organismos (Sheng et al., 2008), de los cuales el succínico, cítrico, glucónico, α-cetoglucónico y oxálico son los más abundantes (Menna et al., 2014).

Con estos mecanismos de acción de los microorganismos solubilizadores de K, además de proveer un potencial biofertilizante, sirven para la bioactivación de las reservas de K del suelo el cual es un tema que requiere ser investigado con el fin de resolver la escasez de fertilizante para la producción de alimentos (Menna et al., 2015).

Géneros y/o especies estudiados. Dentro de las bacterias que solubilizan K existe una amplia gama de bacterias que han sido reportadas; *Pseudomonas*, *Ferrooxidans, Bacillus mucilaginosus, Bacillus edaphicus, B. circulans y Paenibacillus sp* (Sheng, 2005; Lian, et al., 2002; Li et al., 2006; Liu et al., 2012), *Bacillus mucilaginosus, Azotobacter chroococcum* y *Rhizobium* (Singh et al., 2010).

La inoculación con microrganismos solubilizadores de K en el suelo, ha demostrado que mejora la solubilización del K mineral insoluble lo que se traduce en mayores rendimientos en los cultivos. Aunque los microorganismos solubilizadores de K son abundantes en muchos de los suelos, en la actualidad no se ha comercializado con éxito y por lo tanto su aplicación todavía es limitada (Menna *et al.,* 2015).

1.5. Uso de PGPR como biofertilizantes

Los biofertilizantes son considerados dentro del grupo de inoculantes microbianos (Bashan y Holguin 1998) y también pueden ser referidos como bioestimulantes (Calvo et al., 2014). Los biofertilizantes según Vessey (2003) son productos biológicos que contienen microorganismos vivos que, cuando se aplican a la semilla, las superficies de plantas o en el suelo, promueven el crecimiento por varios mecanismos tales como el aumento del suministro de nutrientes, el aumento de la biomasa de raíces o zona de la raíz, y el aumento de la capacidad de absorción de los nutrientes de la planta; según Fuentes-Ramírez y Caballero-Mellado (2005) como productos que contienen microorganismos vivos, los cuales ejercen un efecto benéfico en las plantas, utilizando diferentes mecanismos.

Las PGPR pueden clasificarse como biofertilizantes cuando actúan como fuente de nutrición vegetal y fuente de enriquecimiento para reponer o reconstruir el ciclo de nutrientes entre el suelo, las raíces de las plantas y los microorganismos presentes (Vejan et al., 2016).

Las PGPR debido a sus características, son microorganismos que se han estudiado a profundidad para su uso en biofertilizantes, realizado varios esfuerzos para formularlos y utilizarlos de forma comercial (Kloepper et al., 1989; Fuentes-Ramírez y Caballero-Mellado, 2005). El uso de PGPR en biofertilizantes comerciales comenzó en

los años 1930-1940 con el uso de *Rhizobium*, *Azotobacter* y *Azospirillum* por la empresa “The Nitragin Company” de Milwaukee, Wisconsin, USA.

Existen reportes de que las PGPR han sido utilizadas en todo el mundo como biofertilizantes y que estos han contribuido al aumento de los rendimientos de los cultivos y a la fertilidad del suelo (Khalid *et al.,* 2009). En Rusia y Europa del Este se utilizó a gran escala *Azotobacter* y *Bacillus megaterium*. En la antigua Unión Soviética el biofertilizante fosfobactericina *(Bacillus megaterium var.phosphaticum)* impregnado con Kaolinita llevó a incrementos de hasta 70 % en la producción de los cultivos. También se obtuvieron resultados positivos en suelos de la India deficientes en P, pero fueron negativos en los Estados Unidos (Yarzabal, 2010). En Cuba, Fosforina® es un bioinoculante a base de *Pseudomonas fluorescens* aplicado principalmente en tomate (Uribe et al., 2010). La penetración y aceptación del uso de biofertilizantes ha sido tal que, en países como Brasil y Cuba, la soya es cultivada con biofertilizantes que contienen microorganismos fijadores de nitrógeno sin la utilización de fertilizantes químicos nitrogenados (Bashan, 2008) y en otros cultivos han reducido la aplicación de fertilizantes de síntesis química (N en forma de urea) hasta en un 50 %, (Bonilla y Morales, 2005).

El potencial de las PGPR contribuye al desarrollo de una agricultura sostenible (Khalid et al., 2009) por lo que su aplicación en los sistemas productivos constituye una alternativa viable para reducir los costos de producción y el impacto ambiental asociado a la fertilización química (Alfonso et al., 2005).

1.5.1. Factores determinantes en la efectividad de los biofertilizantes

Durante el desarrollo de inoculantes microbianos efectivos, se deben considerar varios factores. Por ejemplo, la especie y la variedad de plantas pueden a veces ser un factor determinante en la obtención de beneficios utilizando biofertilizantes (Remans et al., 2008). Diferentes especies vegetales o cultivares pueden producir diferentes tipos de exudados radiculares, que apoyan la actividad de los microorganismos inoculados y también sirven como sustratos para la formación de sustancias biológicamente activas por los microorganismos (Khalid et al., 2004). La reproducibilidad de los efectos de los inoculantes microbianos debe ser probada en

una variedad de tipos de suelo y condiciones ambientales. Otro factor clave para el desarrollo de inoculantes microbianos es la formulación comercial (Bashan et al., 2014). Los microorganismos inoculados deben sobrevivir en la formulación seleccionada y producir la actividad deseada después de la inoculación en el campo. Además, cuando se usan en la agricultura convencional, los microorganismos también deben ser compatibles con fertilizantes y productos químicos de protección de cultivos utilizados de forma estándar en semillas o follaje del cultivo objetivo.

La mayoría de los bioinsumos, incluidos los biofertilizantes, ofrecidos a nivel comercial enfocan su uso para cultivos en general, sin necesariamente haber probado su efectividad y/o la adaptabilidad de estos a la rizósfera de los cultivos a tratar, lo que es un factor clave para el éxito en el uso de microorganismos rizosféricos (Lucy et al., 2004). Para obtener un buen resultado en la efectividad de los biofertilizantes se deben utilizar para su elaboración cepas nativas que estén adaptadas a un ambiente y cultivo determinado ya que la efectividad de los biofertilizantes es afectada por diversos factores como humedad, predación, alta salinidad, pH y temperatura (Ferrera-Cerrato y Alarcón; 2001 y Ferrera-Cerrato y Alarcón, 2007).

1.5.2. Uso de bifertilizantes en Chile

En el mercado de insumos agrícolas en Chile, se observa cada vez con más frecuencia la inclusión de bioinsumos para biofertilizantes, sin embargo el 80 % de estos corresponde a insumos importados (SAG, 2014) que no necesariamente han sido previamente probados en los cultivos en los cuales se están aplicando y tampoco se encuentran necesariamente adaptados a las condiciones climáticas de producción del país (Chiang et al., 2013), por lo que existe una falta de conocimiento en la efectividad de las cepas nativas (PGPR) que estén adaptadas al ambiente y los cultivos de importancia económica en Chile.

1.6. El cultivo de tomate (*Lycopersicon sculentum* L.) en Chile

En Chile el tomate se cultiva desde la Región de Arica y Parinacota hasta la Región de la Araucanía, en una superficie de 5.463 hectáreas en el ciclo de producción 2012 (ODEPA, 2013). El 66 % de la superficie nacional con tomate para consumo fresco se concentra entre las regiones de Valparaíso y del Maule. La región con mayor superficie de esta hortaliza es la Región del Maule, con 1 010 hectáreas (19 %), seguida de la Región de Valparaíso, con 966 hectáreas (18 %). En tercer lugar, está la Región Metropolitana, con 867 hectáreas (16 %) (ODEPA, 2013). La producción chilena de tomates para consumo fresco se estima cercana a 300.000 toneladas (Tapia, 2008).

En Chile, el tomate es la hortaliza de mayor consumo y más importante en la alimentación de las familias chilenas (ODEPA, 2013). Con base en datos del INE (2006-2007), el tomate ocupa el primer lugar dentro de las hortalizas de la canasta (ponderación de 0.32 %), lo que significa que es la hortaliza a la que los hogares destinan más recursos económicos. En Santiago se gasta un promedio mensual de $ 2020 en tomate, que corresponde a 0.27 % del gasto total, y en otras ciudades del país $1976, alcanzando un 0.29 % del gasto total (ODEPA, 2013). Consumiéndose internamente el 99.9 % de 300.000 toneladas de tomates para consumo fresco de la producción chilena (Tapia, 2008).

1.7. Uso de las PGPR en la nutrición mineral y crecimiento del tomate

En esta hortaliza en especial, existen diversos estudios donde el uso de PGPR es usado como bioestimulante, incluyendo su capacidad de biofertilizante y promotor de crecimiento, mostrando excelentes resultados en la producción de ácido indolacético, incremento en la longitud de brotes y raíces, cuando fueron aplicadas a la semilla mejoraron la germinación en comparación con semillas no tratadas con inoculaciones de cinco aislados del género *Bacillus* (Agraval y Agraval, 2013). *Bacillus subtilis* BEB-13bs, aplicada en el postrasplante, aumentó el peso seco de las raíces de tomate (18 – 26 %) y la longitud de la raíz (13 – 15 %) significativamente en comparación con el control no tratado. Además, en los frutos de plantas tratadas con

la PGPR se observó un aumento de la longitud del fruto y el peso fresco en 18 % (Mena-Violante y Olalde-Portugal, 2007), mayor firmeza del pericarpio y rendimiento por planta (Mena-Violante et al., 2009) comparado con frutos de plantas no tratadas. Kokalis-Burelle et al., (2002) observaron que en plántulas de tomate provenientes de almacigueras tratadas con PGPR, y trasplantadas a campo, el vigor de las plántulas y la sobrevivencia fue mayor. Santillana et al., (2005) evaluaron 19 cepas del género *Rhizobium* aisladas de raíces de diferentes leguminosas y de distintas regiones, en el crecimiento y germinación en tomate. De las 19 PGPR, siete estimularon el crecimiento de las plantas, nueve promovieron la germinación de las semillas y únicamente dos de esas cepas presentaron efecto positivo en la germinación.

Potasio. Con respecto a nutrición mineral, se realizó un estudio para la evaluación de la inoculación de tomate (*Lycopersicon esculentum* L. F1 híbrido, GS - 15) con PGPR *(Pseudomonas* + *Azotobacter* + *Azosprillum)* y micorrizas arbusculares (AMF) en calidad de la fruta. Los resultados indicaron que el uso de las PGPR + AMF, presentan mayores cantidades de potasio, licopeno y actividad antioxidante en el fruto, mostrando una correlación positiva de $r= 0.86$, $p <0.01$ entre el contenido de potasio y licopeno en el fruto. (Ordookhani et al., 2010). En otro estudio se encontró que *Bacillus pumilus* incrementa de la solubilidad del K^+ y la actividad la actividad antioxidante inducida por las plantas de tomate, particularmente en las enzimas superóxido dismutasa (SOD) y catalasa (CAT) cuando se encuentran Boro-estresadas, lo que ayuda a las plantas de tomate a mantener sus rendimientos bajo este estrés (Sirajuddin et al., 2016).

Nitrógeno. Géneros como *Burkholderia* ha mostrado la capacidad para fijación biológica de N_2 y la promoción del crecimiento vegetal en el cultivo (Caballero-Mellado et al., 2007). Alfonso y Leyva, (2006) realizaron un estudio en la variedad “Amalia”, la cual inocularon con *Glomus clarum y Azospirillum brasilense*, como complemento de la fertilización química nitrogenada, encontrando que la inoculación de PGPR es una alternativa para mantener o sustituir la fertilización nitrogenada, al observar mejores resultados en el tamaño de la planta a partir de los 31 días de la germinación y mayores cantidades de materia seca en las plantas, contenidos de proteínas solubles y rendimiento. En 2010, estudios con la misma variedad de tomate mostraron que

productos comerciales como AzoFert® (*Azospirillum* sp.), combinado con 120 kg de N, produjo un estímulo positivo en rendimiento, 11 % mas que el testigo. Este resultado permite además la disminución de 30 kg de N ha^{-1} en el cultivo, demostrándose así la eficiencia de esta rizobacteria, a partir de una sustitución del fertilizante, que representa un 20 % menos de la cantidad que se aplica según la norma técnica del cultivo en Cuba (Alfonso, 2010).

Fósforo. La bacteria *Bacillus circulans* CB7 en un estudio en tomate bajo invernadero, logró un notable aumento en la germinación de las semillas (22.32 %), en la longitud de los brotes vegetativos (15.91 %) y de raíz (25.10 %); en el peso seco aéreo (52.92 %) y en el de la raíz (31.4 %); en el contenido de N (18.75 %), K (57.69 %) y P (22.22 %) en comparación con plantas sin inocular (Mehta et al., 2013).

En otro un ensayo de campo en donde se evaluó la respuesta de 10 variedades de tomate abierto polinizado (OP) y híbrido a la inoculación de la PGPR, *Bacillus subtilis* bajo dos niveles de fertilización, se encontró que la inoculación de PGPR aumentó significativamente la concentración de P de las variedades evaluadas de 0.20 a 0.21 % P, con un aumento del 10 % en la absorción de P entre las plantas inoculadas. La fertilización de los tomates con la dosis completa de fertilizante recomendada aumentó el rendimiento en un 28 % con respecto a la cosecha obtenida de las plantas aplicadas con la mitad de la dosis recomendada. El ensayo también mostró que los híbridos tienen mayor rendimiento que las variedades OP (Delfin et al., 2015).

El futuro del uso de bacterias PGPR como ingredientes activos de biofertilizantes, tiene un gran potencial para poder ser utilizado en la sustitución parcial de los fertilizantes químicos y puede marcar una importante pauta en la recuperación de la fertilidad del suelo, tanto química, biológica y física. Por lo que la trasferencia de conocimiento y tecnología de las investigaciones en estas rizobacterias a productores agrícolas y el continuo desarrollo de investigación podrán aportar en un futuro cercano, tecnologías mas eficientes, adaptada a diversas condiciones agroecológicas, para la producción de tomate en Chile de manera sustentable con el ambiente.

En base en los estudios antes mencionados sobre el uso de Rizobacterias como insumo para el uso de biofertilizantes en el cultivo de tomate la importancia alimenticia,

nutricional y económica de este cultivo en Chile y la necesidad que existe de generar conocimiento en el uso de cepas nativas de Rizobacterias, se plantean las siguientes hipótesis y objetivos.

1.8. Hipótesis

- Existen cepas nativas de PGPR que inducen mayor crecimiento y contenido de N, P, K en la planta, permitiendo disminuir las aplicaciones de fertilizantes químicos.
- Las bacterias que solubilizan P y K provocan cambios en el equilibrio de las distintas formas de estos elementos en el suelo.

1.9. Objetivo

Seleccionar rizobacterias promotoras del crecimiento vegetal nativas, que fijen N_2 y solubilicen fósforo (P) ó potasio (K) de minerales del suelo, para su uso como biofertilizantes en el cultivo de tomate.

1.9.1. Objetivos específicos

1. Evaluar y cuantificar la capacidad de aislados nativos de rizobacterias para fijar N_2 y solubilizar Fósforo (P) y Potasio (K) *in vitro.*
2. Evaluar la capacidad endofítica y la compatibilidad de las PGPR seleccionadas e identificarlas a nivel de especie.
3. Evaluar el efecto de la inoculación de las PGPR en el crecimiento y estado nutricional, en plántulas de tomate.
4. Conocer el efecto de la inoculación de las PGPR en la disponibilidad de N P K en un suelo con baja fertilidad.

1.10. REFERENCIAS

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II. CAPITULO 2: SELECCIÓN *IN-VITRO* DE RIZOBACTERIAS FIJADORAS DE NITRÓGENO Y SOLUBILIZADORAS DE FÓSFORO Y POTASIO

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2.1 RESUMEN

Las rizobacterias son microorganismos capaces de asimilar formas no disponibles de nutrientes para la planta y transformarlos hasta la obtención de formas asimilables. El objetivo de esta investigación fue seleccionar rizobacterias nativas de Chile por su capacidad de fijar N_2 y solubilizar Fósforo (P) y Potasio (K) *in vitro*. Se evaluaron 72 cepas de una colección de bacterias aisladas de la rizósfera de 10 cultivos de importancia económica en Chile, de las cuales 66 rizobacterias fueron capaces de fijar N_2 en el medio de cultivo Burk's, libre de N y 52 cepas solubilizaron P y K en medio de cultivo Pikovskaya con $Ca_3(PO_4)_2$ y en medio de cultivo Aleksadrov con residuos de feldespato-K (14 % K), respectivamente. Con base en el análisis de conglomerados (correlación cofenética > 0.8), se seleccionaron 20 rizobacterias por su capacidad de fijar N_2, 30 por su capacidad de solubilizar P y 8 por su capacidad de solubilizar K. Se clasificaron como rizobacterias élite a las cepas; Tmt1-107 por su capacidad de fijar N_2 y solubilizar P y K; Brs-127, Hb-142, Ltj-6, T-07, T-72, T3bF, Tmt-15, 19b, por fijar N_2 y solubilizar P; LsC-21, LsC-54, T-06, Tmt-32 y 35b por su fijar N_2 y solubilizar K, *in vitro.* Estas rizobacterias fueron aisladas de plantas de tomate, nabo, haba, lenteja, trigo, ají y lechuga, cultivadas en suelos Andisoles y Ultisoles. El potencial de estas 14 rizobacterias endémicas de Chile para su uso como

biofertilizante, puede contribuir en un futuro a la reducción del uso de fertilizantes químicos y respaldar una producción más sustentable.

Palabras clave: Feldespato-K, PGPR, bacteria diazotrófica.

2.2 INTRODUCCIÓN

La agricultura desempeña un papel vital en el suministro de alimentos. Se espera que la población mundial llegue a 9.730 millones de personas para 2050, lo que significa que la agricultura tiene que producir más alimentos para satisfacer esta creciente demanda y, por lo tanto, mejorar la productividad agrícola (Muller et al. 2017). El nitrógeno (N), el fósforo (P) y el potasio (K) son los principales macronutrientes esenciales para el crecimiento y la productividad de las plantas; sin embargo, no siempre están disponibles para las plantas en el suelo (Meena et al., 2014).

Los diferentes géneros bacterianos son componentes vitales de los suelos. Están involucrados en diversas actividades bióticas del ecosistema del suelo haciéndolo más dinámico y sostenible para la producción de cultivos (Camelo et al., 2011). Las bacterias estimulan el crecimiento de las plantas mediante la movilización de nutrientes en los suelos (Rajkumar et al., 2010). Las bacterias que se alojan alrededor de las raíces de las plantas, las rizobacterias, son versátiles en la transformación, movilización, solubilización de los nutrientes (Hayat et al., 2010). Por ende, las rizobacterias son las fuerzas dominantes en el reciclado de los nutrientes del suelo y, en consecuencia, son cruciales para su fertilidad (Pii et al., 2015). Su aplicación en los sistemas productivos constituye una alternativa viable para reducir los costos de producción y el impacto ambiental asociado a la fertilización química (Alfonso et al., 2005). Con base en lo anterior, el objetivo de esta investigación fue seleccionar rizobacterias nativas de Chile por su capacidad de fijar N_2 y solubilizar fósforo y potasio *in vitro*.

2.3 MATERIALES Y MÉTODOS

Se estudiaron 72 aislados bacterianos de la Colección de Microorganismos de la Facultad de Agronomía de la Universidad de Concepción. Estas bacterias fueron

aisladas de la rizósfera de Ají, Alfalfa, Apío, Girasol, Haba, Lechuga, Lenteja, Tomate y Trigo, cultivados en suelos de tipo Andisol, Alfisol y Ultisol de las Regiones del Maule, Ñuble y BioBío. Los aislados se reactivaron en agar nutritivo estándar.

Para la selección de las rizobacterias, se realizaron tres ensayos en condiciones *in vitro*: (1) Fijación N_2, (2) Solubilización inorgánica P y (3) Solubilización K de minerales. Para cada experimento se utilizó un medio de cultivo específico: Burk's, medio libre de nitrógeno (Hartono et al., 2016); Pikovskaya, PVK (Hariprasad y Niranjana, 2009) y Aleksadrov modificado, MAMs (Meena et al., 2015), respectivamente. En el medio MAMs se preparó con polvo K-feldespato (14 % K), el cual se sumergió en agua durante 48 h para eliminar K soluble, y pH 7.2 ajustado con NaOH 1N.

Se colocaron 15 ml de medio de cultivo específico en cada placa Petri. Posteriormente, en cada placa petri se inocularon 20 aislados más un control negativo (solución salina). Cada aislado se inoculó por triplicado, colocando 3*µl* de cultivo bacteriano (densidad óptica (DO) 600 nm = 1.0) suspendido en NaCl 0.89 % (w/v). Después de la inoculación, las placas petri con medio Burk's y PVK fueron incubadas a 25°C por 72 h y las placas con medio MAMs a 28 ± 1 °C por 7 días.

La fijación N_2 se evaluó midiendo el crecimiento de la colonia bacteriana en medio libre de N (Burk's). Se utilizó una escala numérica para el crecimiento del medio de cultivo de Burk, donde 3 se consideró un crecimiento abundante; 2, buen crecimiento; 1, crecimiento moderado; y 0 (cero), sin crecimiento visible. Para evaluar la solubilización P y K en PVK y MAMs, se midió el diámetro (mm) del halo de solubilización (zona translúcida producida alrededor de cada colonia) utilizando un vernier digital

Para la selección de bacterias, en el ensayo de nitrógeno se seleccionaron las que presentaron un crecimiento de la colonia excelente. Los datos obtenidos en laboratorio de los ensayos de fósforo y potasio fueron sometidos a un análisis de conglomerados (average linkage, datos estandarizados) utilizando, Euclidea como medida de distancia, realizado en el programa InfoStat/E 2013.

2.4 RESULTADOS Y DISCUSIÓN

Fijación de N_2, con base en los datos de crecimiento de la colonia en medio de cultivo libre de N, Agar-Burks, 66 de 72 aislados bacterianos fueron capaces de fijar N_2 *in vitro*; 7 tuvieron crecimiento moderado (1.0), 39 buen crecimiento (2.0) y 20 un excelente crecimiento (3.0). Por lo que estas cepas bacterianas debieron fijar el N_2 contenido en el aire de la placa petri y utilizarlo para su metabolismo. La fijación del N_2 es un proceso de reducción que convierte el nitrógeno molecular (N_2) en amoniaco (NH_3^-) mediante la actividad del complejo enzimático, nitrogenasa (Carvalho et al., 2014). El complejo enzimático nitrogenasa está constituido por dos metaloproteínas: la proteína (I), llamada hierro-molibdeno-proteína, y la proteína (II), llamada hierro-proteína; la enzima requiere de la colaboración de otras dos proteínas: ferrodoxina y avodoxina, que actúan como donadores de electrones y reductores naturales de la nitrogenasa. Los electrones son transportados a la nitrogenasa por la ferrodoxina y llegan a la hierro-proteína, ésta activa a la Mo-Fe-proteína y se produce la reducción de N_2, siendo luego fijado como compuesto aminado (Hoffman et al., 2014).

Este ensayo *in vitro* de fijación de N_2, permitió seleccionar a 20 potenciales fijadoras de N que fueron clasificadas con excelente crecimiento. Estas cepas fueron aisladas de los cultivos: ají, Aj-19b, Aj-88; apio, Ap-3bF, Ap-81b; brócoli, Brs-127, Brs-67; girasol, GE-11; haba Hb-142; lechuga, Ls-C27, Ls-C49, Ls-C54; lenteja, Ltj-6; trigo, T-04, T-06, T-07, T-35b y tomate, Tmt-15, Tmt-31, Tmt-40, Tmt1-107. La fijación biológica de N_2 reduce el problema de perdida de N en comparación con los fertilizantes de N reactivo, ya que al producirse dentro de organismos vivos el N fijado es asimilado rápidamente en constituyentes celulares (Robertson and Groffman, 2015). Poniendo en ventaja el uso de fijadores de N_2 en biofertilizantes comparado con los fertilizantes de N reactivo (Carvalho et al., 2014).

En los cereales, las estimaciones de fijación biológica N_2 por bacterias de vida libre generalmente oscilan entre 5 y 50 kg N ha^{-1} $año^{-1}$ en suelos enriquecidos con residuos vegetales, en sistemas naturales el rango es inferior a estas estimaciones (Roper y Gupta, 2016). Se conoce un gran número de bacterias de vida libre o asociativas que fijan N_2, algunas de las que destacan por su potencial como

biofertilizantes son; *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Derxia*, *Pseudomonas*, *Herbaspirillum*, *Burkholderia*, *Gluconacetobacter*, *Derxia*, *Beijerinckia* and *Azotobacter*, *Enterobacter*, *Bacillus*, *Alcaligenes*, *Klebsiella*, *Lysobacter* sp. and *Paenibacillus polymyxa* (Majeed et al., 2018).

Solubilización de fósforo, en este ensayo 52 de las 72 cepas bacterianas solubilizaron P inorgánico $Ca_3 (PO_4)_2$, presentando halo de solubilización, en un rango de 0.07 a 7.48 mm. Para la selección de las rizobacterias solubilizadoras de P, se agruparon los 72 aislados en tres conglomerados (CLM) de 7 (CLM1), 42 (CLM2) y 23 (CLM3) aislados (correlación cofenética = 0.88) (Figura 1a). El CLM2 presentó los menores halos de solubilización de P, con un promedio de 0.39 mm, seguido del CLM3, con 2.83 mm. El CLM1 agrupó los aislados con mayor halo de solubilización, con un promedio de 5.89 mm (Figura 1b).

Con base en lo anterior, se seleccionaron como posibles solubilizadoras de fósforo las 7 rizobacterias del conglomerado 1; Grs-42 (6.08 mm), Tmt-16 (5.56 mm), Tmt-32 (7.48 mm), Ls-C21 (5.40 mm), Ls-C58 (5.09 mm) Aj-19b (5.56 mm) y Ltj-62 (5.45 mm). Estas bacterias fueron aisladas de la rizósfera de los cultivos de tomate, lechuga, ají, girasol y lenteja. La solubilización de P inorgánico a partir del $Ca_3(PO_4)_2$ en el medio de cultivo Pikovskaya, ocurrió debido a que las bacterias secretaron ácidos orgánicos e inorgánicos como los ácidos; cítrico, oxálico, malónico, láctico, succínico y glucónico. Los grupos hidroxilo y carboxilo de los ácidos quelatan el cation (Ca^{2+}) y disminuyen el pH liberando los fosfatos. En el suelo es similar la solubilización de P mineral, la producción y liberación de ácidos orgánicos por las riozobacterias a la rizósfera provoca su acidificación y esto directamente incrementa la solubilización del P en el suelo (Hariprasad y Niranjana, 2009; Behera et al., 2014).

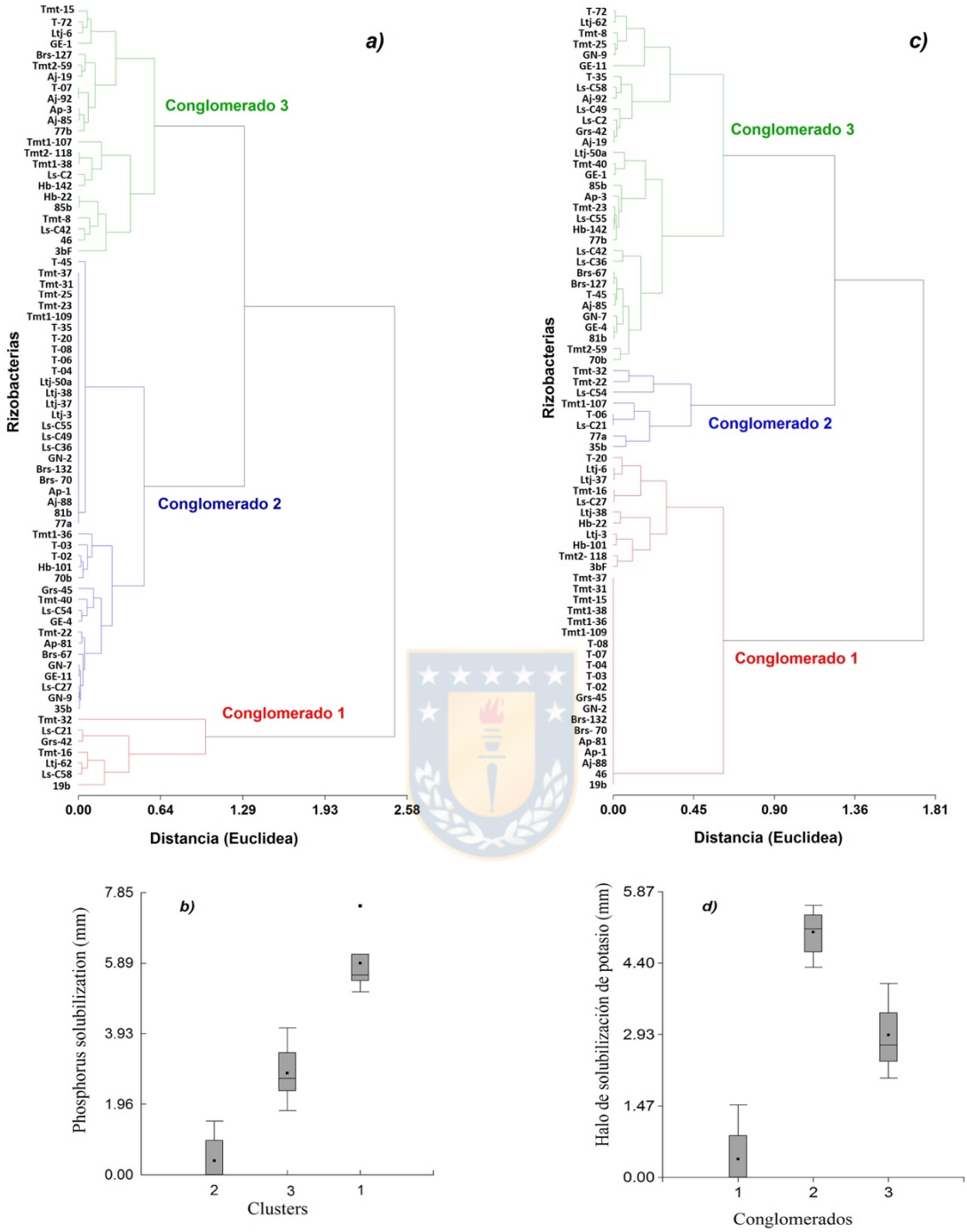
La solubilización de P inorgánico insoluble ayuda a que el P se encuentre una forma disponible para planta. Las diversas formas de suelo P se pueden clasificar como; P en solución, P orgánico insoluble y P. inorgánico insoluble. Los suelos son a menudo altos en fosfatos minerales insolubles pero deficientes en ortofosfatos solubles, esenciales para el crecimiento de la mayoría de las plantas. Por lo tanto, P se suministra a menudo en forma de fertilizantes ($H_2PO_4^-$, HPO_4^{-2}). Sin embargo, las

reacciones en el suelo como la fijación y la inmovilización convierten el P aplicado en formas no disponibles para la planta. Los fertilizantes fosfáticos forman complejos metálicos con Ca^{2+} en suelos calcáreos y con Fe^{3+} y Al^{3+}en suelos ácidos (30 % de la superficie de la tierra). Esto significa que más del 70 – 90 % de la P aplicada se inmoviliza (fijado en las arcillas) en el suelo (Jorquera et al. 2008).

Solubilización de potasio, de un total de 72 aislados evaluados, 52 aislados bacterinaos solubuilizaron K desde feldespato-K, al presentar el halo de solubilización en el medio de cultivo MAMs. Para seleccionar los mejores aislados, se agruparon los 72 aislados bacterianos en tres conglomerados (correlación cofenética = 0.80), formados por 31 (CLM1), 8 (CLM2) y 33 (CLM3) asilados (Figura 1c). El CLM1 (0.37 mm) y el CLM3 (2.93 mm) presentaron halos de solubilización menor a 3.0 mm y el CLM2 presentó halos de solubilización de mayor diámetro, 5.04 mm en un rango de 4.3 a 5.6 mm (Figura 1d).

Existen reportes de halos de solubilización en MAMs de 1.29 a 2.34 cm por cepas aisladas de maíz en India, en donde reportan que las cepas bacterianas aisladas de gramíneas tienden a solubilizar más que las aisladas de las leguminosas (Meena et al. 2015). Sin embargo, en nuestros resultados no existió esta tendencia. Los asilados del trigo (1.92 mm) presentan valores similares a los aislados de lenteja (1.76 mm), haba (1.37 mm) y alfalfa (1.76 mm).

Con base en lo anterior, se seleccionaron los ocho aislados del CLM2: Tmt1-107, T-06, Ls-C21, T-35b, Ap-77a, Tmt-22, Tmt-32 y Ls-C54, los cuales presentaron un halo de solubilización de K (mm) de 5.59, 5.39, 5.39, 5.17, 5.05, 4.78, 4.63, respectivamente. Estas bacterias fueron capaces de solubilizar el K del feldespato-K, al reducir el pH del medio de cultivo y romper la estructura de las micas para satisfacer sus requerimientos de Si^{4+} y K^{+}, inoculaciones con rizobacterias en medio MAM´s con muscovita y biotita han logrado disminuir el pH hasta 4.5 (Meena et al., 2014).



Fuente: Elaboración propia.

Figura 1. Fenograma del análisis de conglomerados de las 72 rizobacterias en base al halo de solubilización (mm) y comparación entre conglomerados: a) y b) solubilización de P inorgánico $Ca_3 (PO_4)_2$ en medio de cultivo Pikovskaya, PVK; c) y d) solubilización de K desde feldespato-K en medio de cultivo Aleksandrov modificado, MAMs.

Las bacterias solubilizadoras de K pueden producir varios tipos de ácidos orgánicos; succinico, cítrico, glucónico, α-ketogluconico y oxálico, e inorgánicos; acetato, citrato, oxalato. Los cuales, al liberarse a la rizósfera, provocan que el K fijado por los minerales sea liberado y disuelto mediante los procesos de acidólisis, quelación y complexólisis. La solubilización de K varía con la naturaleza de los minerales que contienen K (motmorillonita, kaolinite, K-feldspar, muscovita, biotita, illite, ortolasclas) y de las condiciones aeróbicas del suelo (Meena et al., 2015).

La concentración de K soluble en el suelo es muy baja (1 % a 2 %) ya que el K está presente principalmente en rocas insolubles, minerales de silicato y otros depósitos, por lo que no siempre está disponible para las plantas (Parmar y Sindhu, 2013). La inoculación de rizobacterias solubilizadores de K en el suelo, ha demostrado que mejora la solubilización del K mineral insoluble y resultante en rendimientos más altos en los cultivos (Menna *et al.,* 2015).

La aplicación de microrganismo con el objetivo de mejorar la disponibilidad de nutrientes para las plantas es una práctica importante y necesaria para la agricultura sostenible. Durante las últimas dos décadas, el uso de inoculantes microbianos para la agricultura sostenible ha aumentado enormemente en varias partes del mundo, por lo que la selección *in vitro* de estas rizobacterias con capacidad para fijar N_2, solubilizar P y K, sirve para generar en un futuro el desarrollo de biofertilizantes y contribuir al desarrollo ambiental. Es necesario que se realice su estudio con métodos cuantitativos de fijación de N_2 y solubilización de P y K, pruebas *in vivo* para poder realizar una selección con mayor precisión.

2.6 CONCLUSIONES

De un total de 72 aislados de rizobacterias, 66 fueron capaces de fijar N_2 y 52 de solubilizar P y K. Se seleccionaron 20 rizobacterias por su capacidad de fijar N_2, 30 por su capacidad de solubilizar P y 8 por su capacidad de solubilizar K.

Se clasificaron como bacterias élite a la cepa Tmt1-107 por su capacidad de fijar N_2 y solubilizar P y K, a las cepas, Brs-127, HB-142, Ltj-6, T-07, T-72, T3bF, Tmt-15, 19b, por su capacidad de fijar N_2 y P y a las cepas LsC-21, LsC-54, T-06, Tmt-32 y 35b

por su capacidad de fijar N_2 y solubilizar K, *in vitro,* aisladas de plantas de tomate, nabo, haba, lenteja, trigo, ají y lechuga, cultivadas en suelos Andisoles y Ultisoles.

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III. CAPITULO 3: PLANT GROWTH-PROMOTING RHIZOBACTERIA ABLE TO IMPROVE NPK AVAILABILITY: SELECTION, IDENTIFICATION AND EFFECTS ON TOMATO GROWTH

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3.1 ABSTRACT

Nitrogen, P and K are essential macronutrients that are not readily available to plants. Rhizobacteria are able to convert these unavailable forms for subsequent uptake by the plant, diverse species have been characterized as N_2 fixers, P solubilizers and capable to solubilize mineral K from unavailable forms. The objective of this study was to select rhizobacteria capable of improving NPK availability and promoting tomato (*Solanum lycopersicum* L.) growth. Fifteen strains were studied. Four strains were selected for their capacity to fix N_2, three for their ability to solubilize P, and six for their capacity to solubilize biotite and K-feldspar, isolated from tomato, lentil, chili pepper, faba bean and lettuce crops in Andisol and Alfisol soils. Through 16SrRNA sequencing, selected strains were identified as *Pseudomonas gessardi*, *P. koreensis*, *P. brassicacearum*, *P. marginalis*, *Acinetobacter calcoaceticus* and *Rahnella aquatica*. Phosphorus solubilizing strains did not show a positive effect on plant growth or an increase in available soil P. The N_2 fixing bacteria Tmt-16 strain increased root growth in 23.57 %; maintained the highest N content in plant tissue, 2.60 %, higher amount of N available in the soil, 2.95 mg kg^{-1}, and a higher content of N-NH_4^+ 1.95 mg kg^{-1}. The

K solubilizing strains Ls-C21, Ltj-62 and LsC-58 reached 17.0 to 19.0 mg kg^{-1} of available K and 0.04 to 0.05 mg kg^{-1} of interchangeable K ($p \leq 0.05$.). These four endemic rhizobacteria can be potentially used as biofertilizers, allowing a reduction in the use of chemical fertilizers and a more sustainable production of tomatoes.

Key words: *Acinetobacter*, phosphorus and potassium solubilization, N_2 fixation, *Pseudomonas*, *Rahnella*, *Solanum lycopersicum*.

3.2 INTRODUCTION

Nitrogen, P and K are essential macronutrients for plant growth and crop yield. However, they are not readily available to plants (Meena et al., 2014). The largest reservoir of N is found in the form of atmospheric N (78 %), in a form biologically unavailable to plants (Hartono et al., 2016). Phosphorous in soil is present as P in solution, insoluble organic P and insoluble inorganic P, soils are often high in insoluble mineral phosphates but deficient in soluble orthophosphate, which is essential for the growth of most plants (Hariprasad and Niranjana, 2009). The concentration of soluble K in soil is usually very low (1 % to 2 %) since K is mainly present as insoluble rocks, silicate minerals and other deposits, although it is not always available to plants (Parmar and Sindhu, 2013). Rhizobacteria are able to convert unavailable forms of nutrients into available ones for subsequent uptake by the plant (Meena et al., 2014).

Plant growth-promoting rhizobacteria (PGPR) are soil bacteria that are able to colonize rhizosphere and to enhance plant growth by means of a wide variety of mechanisms. Diverse species PGPR have been characterized as N fixers, P solubilizers and capable to solubilize mineral K from unavailable forms. N_2 fixation occurs when molecular N (N_2) is converted into ammonia (NH_3^-) by the enzyme complex nitrogenase, which consists of two metalloproteins, the molybdenum-iron protein (Protein 1) and the iron-protein (Protein 2). Nitrogenase requires the collaboration of two other proteins: ferrodoxin and avodoxin, which act as electron donors and natural reducers of nitrogenase. The electrons are transported to the nitrogenase by ferrodoxin and reach the iron-protein. This activates the Mo-Fe-protein and the reduction of N_2 occurs, being then fixed as an amino compound (Hoffman et

al., 2014). Solubilization of inorganic P occurs by secretion of organic and inorganic acids, such as citric, lactic, succinic and gluconic acids. Hydroxyl and carboxyl groups of acids chelate cations (Ca^{2+}) and lower the pH by releasing the phosphates (Behera et al., 2014). Potassium-solubilizing bacteria (KSB) can produce several types of organic acids; succinic, citric, gluconic, α-ketogluconic and oxalic, and inorganic; acetate, citrate, oxalate that when released to the rhizosphere cause the fixed K of the minerals to be released and dissolved by processes of acidolysis, chelation and complexolysis. The solubilization of K varies with the nature of the minerals (motmorillonite, kaolinite, K-feldspar, muscovite, biotite, illite, orthoclases) that contain K and aerobic conditions (Meena et al., 2015).

Of the large number rhizobacteria, the following genera have shown potential as biofertilizers or growth promoters: *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Acinetobacter Beijerinckia*, *Derxia*, *Herbaspirillum*, *Burkholderia*, *Gluconacetobacter*, *Enterobacter*, *Bacillus*, *Rahnella*, *Alcaligenes*, *Klebsiella*, *Lysobacter* and *Paenibacillus*. The use of PGPR has proven to be an environmentally sound way of increasing crop yields by facilitating plant growth. It can also reduce production costs and the environmental impact associated with chemical fertilization (Majeed et al., 2018).

Several PGPR have been isolated from different soils and crops in Central Chile and have shown that they can promote root and aerial growth, and increase nodule formation in legumes (Gerding et al., 2017; Sepúlveda-Caamaño et al., 2018; Cedeño-García et al., 2018). The aim of this study was to select rhizobacteria capable of improving NPK availability and promoting tomato growth.

3.3 MATERIALS AND METHODS

3.3.1 Selection of rhizobacteria for N2 fixation, and solubilization of P and K

Fifteen bacterial isolates from the Microorganism Collection of the Faculty of Agronomy were studied. These isolates had been formerly collected from the rhizosphere of nine major agricultural crops in Chile, grown in different soils of the

Maule, Ñuble and Biobío Regions (Table 1). The isolates were reactivated in standard nutrient agar.

The bacteria were evaluated under laboratory conditions for (1) N_2 fixation, (2) P inorganic solubilization, and (3) K solubilization of minerals. Using the specific culture media Burk's, N-free medium (Hartono et al., 2016), Pikovskaya (PVK) (Hariprasad and Niranjana, 2009) and modified Aleksandrov medium (MAMs), waste mica was used biotite (11 % K) and K-feldspar (14 % K) (Meena et al., 2015), respectively.

3.3.2 Capacity of the isolates to fix N_2 and solubilize P and K

The screenings were carried out with three replicates for each of the isolates. Petri dishes with Burk's and PVK were incubated at 25 °C for 72 h, while MAMs was incubated at 28 ± 1 °C for 7 d. The ability of the isolates to fix N_2 and solubilize P and K was evaluated as follows: N_2 fixation was assessed by measuring the growth of the bacterial colony in N-free medium (Burk's). A numerical scale for growth in Burk's culture medium (GBCM) was used, where 3 was considered abundant growth; 2, good growth; 1, moderate growth; and 0 (zero), no visible growth. To evaluate P and K solubilization in PVK and MAMs, the diameter (mm) of the solubilization halo (translucent zone produced around each colony) was measured using a digital vernier (Hariprasad and Niranjana, 2009; Meena et al., 2015). Microphotographs of feldspar-K solubilization in MAMs media were taken from plates with and without bacterial inoculum. The microphotographs were taken at the Center for Mineralogical Studies CEM Geoatacama, with a scanning electron microscope (VEGA3, TESCAN, Kohoutovice, Czech Republic).

Table 1. Host plant, place of collection and production of NPK in mg L^{-1} of the rhizobacteria.

					N_2 Fixation		P Solubilization		K Solubilization	
Soil type	Geographic origin	Host plant	Location in the root	Strain	GBC	NH_4^+	PSH	PO_4	KSH	K
Andisol	36º37'21" S, 71º51'53" W	*Capsicum annuum* L.	Exorhizosphere	Aj-19b	3.0	1.4	5.1	109.2	0.0	24.0
		Lens culinaris Medik.	Endorhizosphere	Ltj-62	2.0	0.2	5.4	120.5	3.8	29.0
		Apium graveolens L.	Exorhizosphere	Ap-3bF	3.0	0.1	3.4	106.7	1.1	15.0
			Exorhizosphere	Ap-77a	2.0	0.1	0.0	160.4	5.1	0.0
		Vicia faba L.	Endorhizosphere	Hb-142	3.0	0.7	3.8	225.1	2.7	0.0
		Solanum lycopersicum L.	Endorhizosphere	Tmt1-107	3.0	0.6	4.1	308.6	5.6	0.0
	36º37'9" S, 71º49'43" W	*Lactuca sativa* var. *capitata* L.	Exorhizosphere	Ls-C54	3.0	0.0	1.2	155.0	4.3	37.0
	36º37'24" S, 71º52'59" W		Endorhizosphere	Ls-C21	1.0	0.0	6.1	144.7	5.4	30.0
	36º38'32" S, 71º51'13" W		Endorhizosphere	Ls-C58	1.0	0.1	5.5	241.2	3.2	25.0
	36°40'24" S, 71°46'48" W	*Triticum aestivum* L.	Exorhizosphere	T-35b	3.0	0.1	1.9	133.5	3.8	20.0
			Rhizoplane	T-06	3.0	0.2	0.0	126.4	5.4	12.0
Ultisol	36°45.78' S, 72°04.8' W	*S. lycopersicum* L.	Exorhizosphere	Tmt-22	2.0	0.1	0.9	181.9	4.8	18.0
			Exorhizosphere	Tmt-16	1.0	1.7	5.5	84.4	1.5	5.0
			Exorhizosphere	Tmt-32	2.0	0.0	7.5	144.4	4.6	26.0
Alfisol	35º57'20" S, 72º17'9" W	*Medicago sativa* L.	Exorhizosphere	GE-11	3.0	0.3	1.0	165.8	3.6	0.0

GBC: Growth in Burk's culture medium, 3 = abundant, 2 = good, 1 = moderate. PSH: Solubilization halo in Pikovskaya medium (PVK, mm). KSH: Solubilization halo in modified Aleksandrov medium (MAMs) (mm).

Fuente: Elaboración propia.

3.3.3 Determination of NPK availability in solution

Burk's, PVK and MAMs liquid media were used to quantify the amount of fixed N and solubilized P and K by the bacterial strains selected. Liquid media were inoculated with each isolate and incubated in a rotary shaker (150 rpm) at 25 °C for 3 d for Burk's and PVK, and MAMs at 28 ± 1 °C for 7 d.

For each evaluation, 20 mL of bacterial suspension prepared with 100 µL of bacterial culture (optical density (OD) 600 nm = 1.0) suspended in 0.89 % NaCl (w/v) were placed in 50 mL sterile falcon tubes. After incubation, the samples were centrifuged (Centrifuge 5804 R, Eppendorf, New York, USA) for 10 min at 11000 rpm; the supernatant was removed with a syringe with a sterile 0.45-µ filter and placed in 16 mL sterile test tubes. Each filtered sample was used to determine NH_4^+, phosphate (PO_4) and K in solution.

The production of N-NH_4^+ (mg L^{-1}) by the selected isolates was assessed by direct Nesslerization (Vyas et al., 2010). For each sample, a volume of 2 mL of filtrate was taken. Then 2 mL Nessler reagent and 3 mL NaOH (3 M) were added, vortexed and allowed to stand for 20 min before reading in the spectrophotometer (Optizen POP-Bio, Mecasys, Daejeon, Korea) at 490 nm. The calibration curve was constructed with N-NH_4^+ at 0, 2, 5, 10, 20 and 30 mg L^{-1}.

The solubilization of P-PO_4 (mg L^{-1}) was assessed by colorimetry with the molybdenum blue method using C_6H_8O as reductant, with modifications (Behera et al., 2014). Samples of the filtrate were diluted 10 times. Volumes of 2 mL of the diluted sample were placed in 100-mL flasks. Then 43 mL distilled water and 5 mL reagent were added to determine phosphate. The mixture was homogenized in an orbital stirrer (Super-Mixer, Lab-Line Instruments, Melrose Park, Illinois, USA) for 5 s and allowed to stand for 30 min to observe color changes to azure blue. The readings of the samples were performed on a spectrophotomete (UVE 054108, Helios Epsilon, Thermo Scientific, Waltham, Massachusetts, USA) at 720 nm. The calibration curve was made with a phosphate standard solution at 1000 mg P-PO_4 L^{-1}, at concentrations of 0.04, 0.08, 0.16, 0.24, 0.32, 0.40 and 0.48 mg P-PO_4 L^{-1}.

The solubilization of K (mg L^{-1}) was determined by atomic emission spectrometry of air-acetylene flame by direct aspiration (Zhang and Kong, 2014). The readings of the samples were performed at a wavelength of 766.5 nm. The calibration curve was performed with a standard solution of 1000 mg L^{-1} in the following concentrations 0.0, 2.5 and 5.0 mg L^{-1}.

3.3.4 Identification of selected bacterial isolates

Cells were prepared for polymerase chain reaction (PCR), by suspending 100 µL of the bacterial culture in 900 µL nuclease-free water and centrifuging at 5000 g for 5 min. The supernatant was removed, and the cell pellet was resuspended in 1 mL nuclease-free water. This procedure was repeated twice and finally the cell concentration was standardized at an OD 600 nm = 0.4 (CE 1020, Cesil Instruments, Cambridge, UK). Finally, the samples were subjected to a temperature shock at 65 °C for 5 min and then at -20 °C for 5 min (Guiñazú et al., 2013).

The 16S rRNA partial gene was amplified through PCR using the universal primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR reaction mix consisted of 2 µL of cell suspension, 2 U Taq DNA polymerase (GoTaq, Promega, Madison, Wisconsin, USA), 0.5 µM of each primer, 1.5 mM $MgCl_2$, 1X GoTaq buffer solution, 0.2 mM dNTPs and ultrapure water Hyclone (Thermo Scientific) for a final volume of 40 µL. The PCR conditions were the following: one initial denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 7 min. The amplification of the gene was verified through electrophoresis in agarose gel 1 % (w/v), stained with GelRed (Biotium, Hayward, California, USA) at 90 V for 45 min (Sepúlveda-Caamaño et al., 2018).

The PCR products were purified and sequenced at Macrogen Inc. (Seoul, Korea; http://dna.macrogen.com/eng). The chromatograms obtained were analyzed and edited in the GeneTool Lite 1.0 (2000) software. The phylogenetic tree was constructed using the Neighbor-Joining algorithm using MEGA 7 software (Tamura et al., 2004). Type strains sequences were obtained from GenBank of the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov).

3.3.5 Endophytic capacity of selected bacterial isolates

Fragments 3 cm long were taken from the distal ends of the roots, stem and leaves of the inoculated plants. Then these sections were washed in distilled water and disinfected superficially in 70 % ethanol for 2 min, followed by immersion in 1 % hypochlorite for 30 s, and four washes in sterile distilled water. Subsequently, the fragments were cut into 1 cm pieces and cultured in standard nutrient agar plates, incubated for 48 h at 25 °C (Parsa et al., 2013). The bacterial colonies developed from the ends of the cuts were re-isolated on standard nutrient agar and subject to the same growth conditions mentioned above. Each treatment was established in triplicate for molecular analysis.

The identity of the isolates obtained was assessed at the strain level by molecular fingerprinting using the primer BOX A1R (5'-CTA CAA CGG GCT GAC GGC GAC G-3') according to the methodology used by Guiñazú et al. (2013). The PCR reaction mix contained 5.0 µL of cell template, 0.3 µL Gotaq polymerase (Promega) (5 U μL^{-1}), 5.0 µL A1R primer (10 µM) 0.5 µL dNTPs (10 mM), 5.0 µL Gotaq buffer (5×) solution, 1.5 µL $MgCl_2$ (25 mM) and 7.7 µL ultrapure water, making a total of 25 µL. The PCR conditions were: 95 °C for 7 min; then 35 cycles at 94 °C for 1 min; 52 °C for 1 min and 72 °C for 8 min; and finally, 72 °C for 16 min with amendments in the time of PCR runs. The PCR products were analyzed by electrophoresis in 2 % (w/v) agarose gels with 2.5 mL^{-1} µL Gel Red (10000× in DMSO, Biotium) in a 1× TAE buffer solution (40mM Tris-acetate, 1 mM EDTA, pH 8.0) at 50 V for 3 h. Bands were visualized in a UV transilluminator.

3.3.6 Effects of the selected bacterial isolates in plant and soil

The capacity of bacteria to promote plant growth in tomato ‘Cal Ace’ was evaluated. Plants were grown in speedlings using soil of the following characteristics as a substrate: 7.0 mg kg^{-1} available N (nitrates N-NO_3 4.90 mg kg^{-1} and ammonium N-NH_4^+ 2.10 mg kg^{-1}), 19.20 mg kg^{-1} available P, 40.90 mg kg^{-1} available K, 5.66 pH in water and 4.18 % organic matter. The soil was classified as Isotic, mesic Dystric Xeropsamments belonging to the Arenales series.

Three experiments were carried out, one for each type of selected bacteria; N_2 fixing bacteria (3 NFB strains + 1 negative control without inoculation), P solubilizers (4 PSB strains+ 1 negative control) and K solubilizers (5 KSB strains + 1 negative control). A completely randomized design was used with three replicates. Each experimental unit consisted of 27 plants.

The experiments were carried out in a phytotron with high-pressure sodium steam lamps (400 W Gro-lux, Osram Sylvania, Danvers, Massachussetts, USA) providing a minimum photosynthetic photon flux density (PPFD) of 400-500 mol m^2 s^{-1}. The temperature ranged from 21 to 27 °C during germination and from 21 to 24 °C during vegetative growth, with a RH between 60 % and 80 %. Planting and nutritional management of the plants was carried out based on Ojodeagua et al. (2008).

Seeds, soil and germination trays were disinfected prior to sowing. The seeds were disinfected with 5 % hypochlorite (W/V) for 3 min and with 70 % ethanol (w/v) for 1 min, followed by six washes in sterile distilled water. The soil was disinfected with steam at an initial temperature of 100 °C and then maintained at 82 °C for 30 min. The germination trays were disinfected with 10 % hypochlorite (w/v).

Plant inoculation. Plants were inoculated twice: at emergency stage and 15 d after emergency. Bacterial concentration for each strain was standardized at 10^7 CFU mL^{-1} in 1.0 % sucrose (v/v) determined through absorbance (λ = 600 nm). For inoculation, 1 mL standardized bacterial suspension was placed aseptically in the root zone of each plant.

Plant growth promotion. At day 35 after planting, plants were removed from the soil and their roots carefully washed. The length (cm) and fresh and dry weight (g) of both roots and aerial shoots were evaluated. Dry weight was determined after roots and shoots were dried in a stove until reaching constant weight (70 ºC/48 h).

NPK determination in plant tissue and soil. NPK were determined through soil analysis methods of the Commission for Standardization and Accreditation of the Chilean Society of Soil Science. In plant the N analysis was carried out by the digestion method and colorimetric determination; in soil inorganic N was determined by colorimetry, nitrates via nitration of salicylic acid, and ammonium using Nessler

reagent. Phosphorous content was determined by calcination and colorimetric determination of the phospho-vanadomolybdate, and K content was determined through calcination and atomic emission spectrometry.

3.3.7 Statistical analysis

Data obtained in laboratory were subjected to cluster analysis (standardized data) using euclidea as a measure of distance. The defined clusters and the bacterial isolates of the best cluster(s) were subjected to a nonparametric variance analysis with the Kruskal Wallis test and a comparison of means test. Data obtained each trial in plant, completely randomized design with three replicates, were subjected to ANOVA and means were compared through the Tukey test. Analysis were realized with a probability of $p \leq 0.05$ using the InfoStat/E 2013 software.

3.4 RESULTS AND DISCUSSION

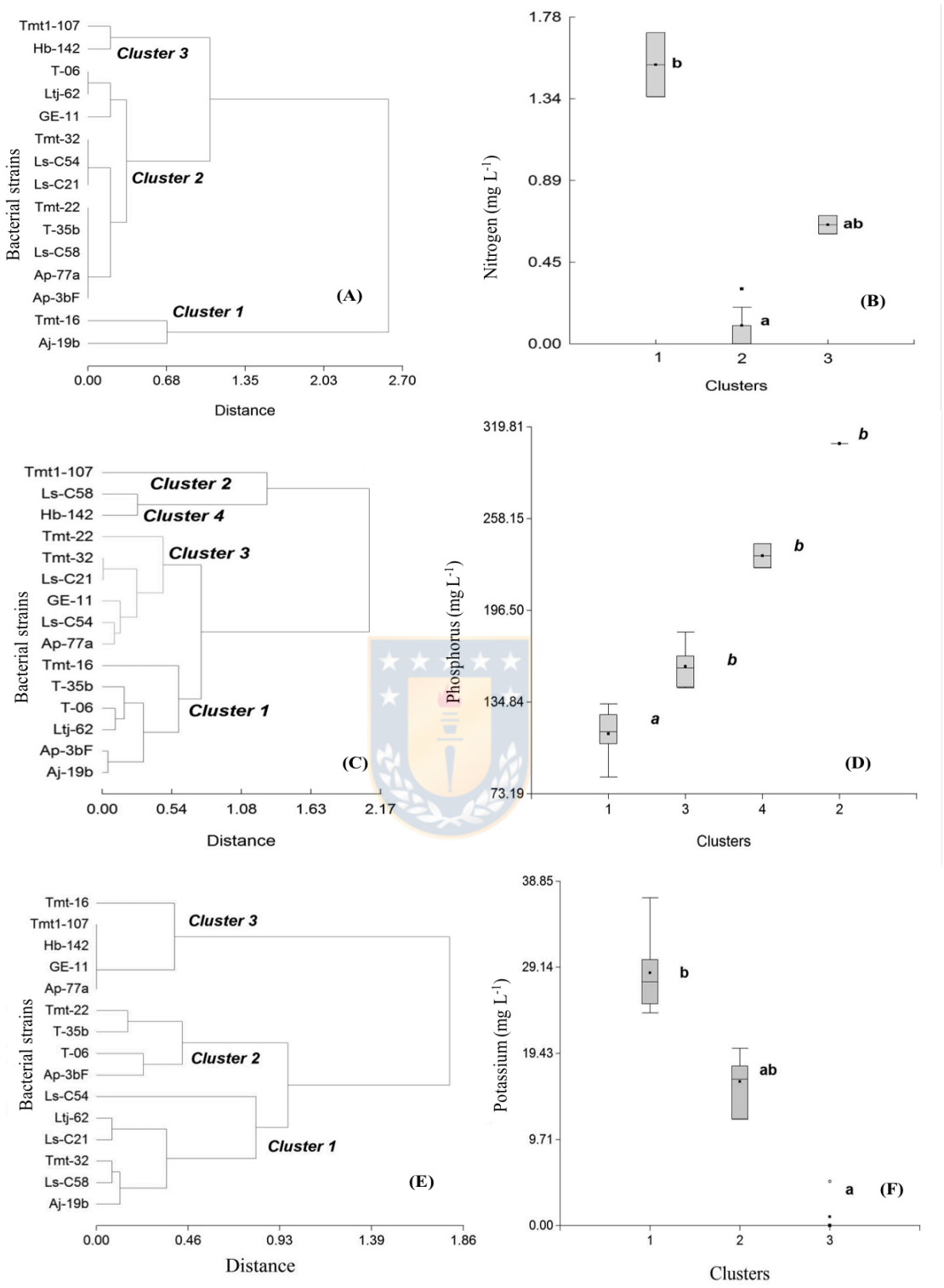
3.4.1 Selected rhizobacteria for N_2 fixation, solubilization of P and K

N_2 fixation and amonium production (N-$NH4^+$). The 15 isolates evaluated had growth of bacterial colony in Burk's agar medium; 8 isolates had abundant growth, 4 had good growth and 3 had moderate growth. Determination of N-NH_4^+ in Burk's liquid medium showed that 12 out of the 15 strains made NH_4^+ available (Table 1). These bacterial strains probably fixed the N_2 contained in the air of the falcon tubes and used it for their metabolism. N_2 fixation occurs when molecular N (N_2) is converted into ammonia (NH_3^-) by the enzyme complex nitrogenase, which consists of two metalloproteins, the molybdenum-iron protein (Protein 1) and the iron-protein (Protein 2). Nitrogenase requires the collaboration of two other proteins: ferrodoxin and avodoxin, which act as electron donors and natural reducers of nitrogenase. The electrons are transported to the nitrogenase by ferrodoxin and reach the iron-protein. This activates the Mo-Fe-protein and the reduction of N_2 occurs, being then fixed as an amino compound (Hoffman et al., 2014). The process of N_2 fixation varies between different bacterial genera. Most of biological N fixation is carried out by the activity of molybdenum nitrogenase, which is found in all diazotrophs (Carvalho et al., 2014).

The concentrations of N-NH_4^+ produced ranged from 0.2 to 1.7 mg L^{-1} (Table 1). Studies on asymbiotic N_2 fixing bacteria (NFB) have reported higher levels of NH_4^+ than the ones on this work, with concentrations of 5.17 mg L^{-1} by *Azotobacter* sp.; 3.65 mg L^{-1} by *Azotobacter vinelandii* DSM 2289; 16.63 to 6.89 mg L^{-1} by *Azotobacter vinelandii* (GMA6 and GMA9); 4.68 mg L^{-1} by *Azospirillum* spp.; 1.948 mg L^{-1} by *Azospirillum brasilense* DSM 1224; 10.34 mg L^{-1} by *Brevibacillus formosus* (GMA3); and 5.88 mg L^{-1} by *Stenotrophomonas* sp. (GMA1) (Hartono et al., 2016).

For the selection of NFB, the 15 strains were grouped into three clusters (CC = 0.96): CL1, CL2 and CL3 (Figure 2A) CL1 presented mean NH_4^+ concentrations of 1.53 mg L^{-1}, producing 1.42 mg L^{-1} more than CL2 (0.11 mg L^{-1} NH_4^+) and 0.88 mg L^{-1} more than CL3 (0.65 mg L^{-1} NH_4^+) (ANOVA, P = 0.002). However, CL3 did not show significant differences with respect to CL1 and CL2 (Figure 2B). Therefore, an ANOVA was performed between CL1 and CL2 isolates: Aj-19b (1.35 mg L-1 $NH4^+$), Hb-142 (0.70 mg L-1 $NH4^+$), Tmt1-107 (0.60 mg L-1 $NH4^+$), Tmt-16 (0.70 mg L-1 $NH4^+$). As no differences were found in terms of $NH4^+$ production (p = 0.40), all isolates were selected for the plant trials.

The four bacterial strains selected as NFB were isolated from the following crops: chili, Aj-19b; faba bean, Hb-142; tomato, Tmt1-107 and Tmt-16. Nitrogen-fixing bacteria have the ability to develop different types of root associations with different plant species (Carvalho et al., 2014).



Average - average linkage, Euclidean distance. Means with the same letter are not significantly different ($p > 0.05$). Fuente: Elaboración propia.

Figure 2. Phenogram of the cluster analysis of the 15 rhizobacteria, and comparison between clusters: (A) (B) NH_4^+ production; (C) (D) P solubilization; (E) (F) K solubilization.

Phosphate solubilization and P production ($P\text{-}PO_4$). Out of 15 isolates evaluated, 12 bacterial isolates presenting solubilization halos on the PVK-agar plate, with a halo from 0.9 to 7.5 mm (Table 1). Solubilization of inorganic P from $Ca_3(PO_4)_2$ in PVK media occurs by secretion of organic and inorganic acids, such as citric, lactic, succinic and gluconic acids. Hydroxyl and carboxyl groups of acids chelate cations (Ca^{2+}) and lower the pH by releasing the phosphates (Behera et al., 2014).

Determination of the available P in solution showed that the 15 strains evaluated were able to solubilize the phosphate of the tricalcium phosphate $Ca_3(PO_4)_2$, ranging from 84.4 to 308.6 mg L^{-1}. Several studies have reported that many isolates do not produce any visible halo zone on the agar plate, but they can solubilize several types of insoluble inorganic phosphate in liquid medium (Behera et al., 2017). This may be due to varying diffusion rates of different organic acids secreted by different microorganisms.

The 15 bacterial isolates were grouped into four clusters (CC = 0.83); CL1, CL, CL3 and CL4 (Figure 2C), consisting of 6, 1, 6 and 2 isolates with average P values of 113.45, 308.60, 158.70, 233.15 mg L^{-1}, respectively. Analysis of variance showed significant differences (P = 0.0177) between CLs. The CL1 recorded the lowest P production in comparison with and thed CL3, CL4 and CL2 (Figure 2D).

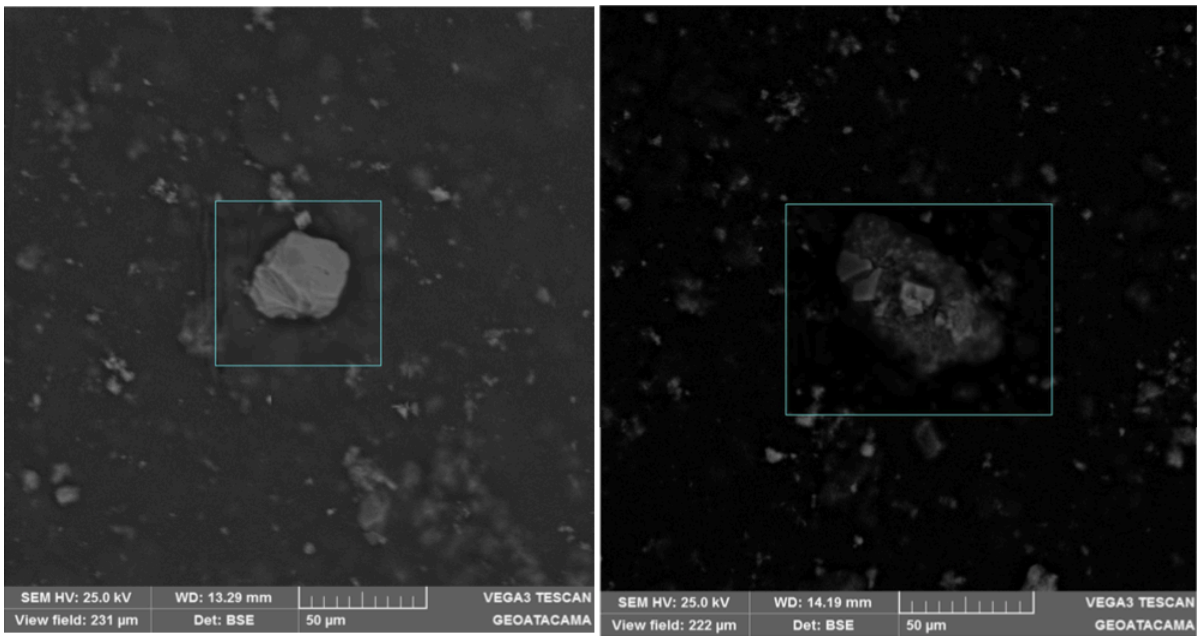
For the plant trials, the following isolates of CL2 and CL4 were selected: Tmt1-107 (308.60 mg $P\text{-}PO_4$ L^{-1}), Ls-C58 (241.20 mg $P\text{-}PO_4$ L^{-1}) and Hb-142 (225.10 mg $P\text{-}PO_4$ L^{-1}). These isolates solubilized phosphorus in larger amounts than those reported in other studies by Ehsan et al. (2016) that determined P solubilization from tricalcium phosphate by species of the genus *Pseudomonas*, *P. beteli* and *P. lini*, which solubilized phosphate in a range from 18.30 to 147.53 µg mL^{-1}. There are reports that indicate that the use of PSB in co-composting of urban waste (95 %) + phosphate rock (5 %) can generate up to 25 kg of phosphorus per 1 t fresh weight ha^{-1} (Naher et al., 2018).

Potassium solubilization and production. Out of 15 isolates evaluated 14 bacterial isolates solubilized K from K-feldspar in MAMs-agar, with a solubilization halo from 1.1 to 5.6 mm (Table 1). Solubilization of K occurs by secretion several types of

organic acids; succinic, citric, gluconic, α-ketogluconic and oxalic, and inorganic; acetate, citrate, oxalate that when released to the rhizosphere cause the fixed K of the minerals to be released and dissolved by processes of acidolysis, chelation and complexolysis. In the Figure 3 shows the effect of a bacterium on K mica (K-feldspar) by breaking down the mineral and resulting in a 5 % solubilization of the K contained in the feldspar. These K-solubilizing bacteria can produce several types of organic acids that possibly break down the structure of the micas to satisfy their Si^{4+} and K^{+} requirements in the culture medium within the plate, consequently reducing the pH of the medium.

Based on a CC = 0.82 in the cluster analysis of the 15 bacterial isolates, three clusters were identified, CL1, CL2 and CL3 (Figure 2E), consisting of 6, 4 and 5 isolates with average K values of 28.5, 16.25 and 1.0 mg L^{-1}, respectively. Analysis of variance showed significant differences (P = 0.0018) between CL1 and CL3; CL1 isolate showed the highest K-solubilizing capacity (Figure 2F). For the plant trials, six CL1 isolates were selected: Ls-C54 (37.0 mg K L^{-1}), Ls-C21 (30.0 mg K L^{-1}), Ltj-62 (29.0 mg K L^{-1}), Tmt-32 (26.0 mg K L^{-1}), Ls-C58 (25.0 mg K L^{-1}), and Aj-19b (24.0 mg K L^{-1}).

The six selected isolates showed K production ranging from 24.0 to 37.0 mg L^{-1} (Table 1). Similar ranges were obtained by Parmar and Sindhu (2013) from 15 to 48 mg L^{-1}, when quantifying the solubilization of K from mica powder by six bacterial strains. These values were lower than those reported for the strain *Bacillus mucilaginosus*, AS1.153 when solubilizing K in motmorillonite, kaolinite and K-feldspar, with values ranging between 90 and 140 mg K L^{-1}. However, these values are higher than those obtained by Zhang and Kong (2014), where 17 isolates of KSB reached from 0.59 to 4.4 mg K L^{-1}, and by *Pseudomonas azotoformans*, which solubilized 6.03 mg K L^{-1} (Saha et al., 2016). The solubilization of K varies with the nature of the minerals (motmorillonite, kaolinite, K-feldspar, muscovite, biotite, illite, orthoclases) that contain K and aerobic conditions (Meena et al., 2015).



Unaltered K-feldspar, 14% K (left) compared to K-feldspar inoculated with illite/muscovite reaction boundary, 9% K, in modified Aleksandrov medium (MAMs) agar (right). Fuente: Elaboración propia.

Figure 3. Microphotographs of feldspar-K solubilization in MAMs media in from plates with and without bacterial inoculum.

3.4.4 Identification of the selected bacteria

For all selected strains, a 1200 bp fragment of the 16S rRNA gene was amplified. The phylogenetic tree based on 16S rRNA grouped the bacterial strains into three genera: *Pseudomonas*, *Rahnella* and *Acinetobacter* (Figure 4).

Of the strains within the *Pseudomonas* genus, Ltj-62 and Aj-19b were closely related to the type strain *P. brassicacearum* DBK11^T (AF100321.1), with a similarity of 99.998 %. Hb-142 was grouped with the type strain *P. marginalis* ATCC 10844^T (AB021401.1), with a similarity of 100 %. Tmt-16 was grouped with *P. gessardi*^{T} (AF074384.1), with a similarity of 99.971 %, and Tmt-32 and Ls-C21 were grouped with *P. koreensis* Ps 9-14^T = LMG 21318^T = KACC 10848^T (NR 025228.1), with similarity scores of 99.998 % and 100 %, respectively.

Bacteria of the *Pseudomonas* genus have been extensively described as PGPR, and endophytes of several plants (Carvalho et al., 2014). Species such as *P. monteilii* and *P. mandelii* have been reported as NFB associated with rice plants (Habibi et al.,

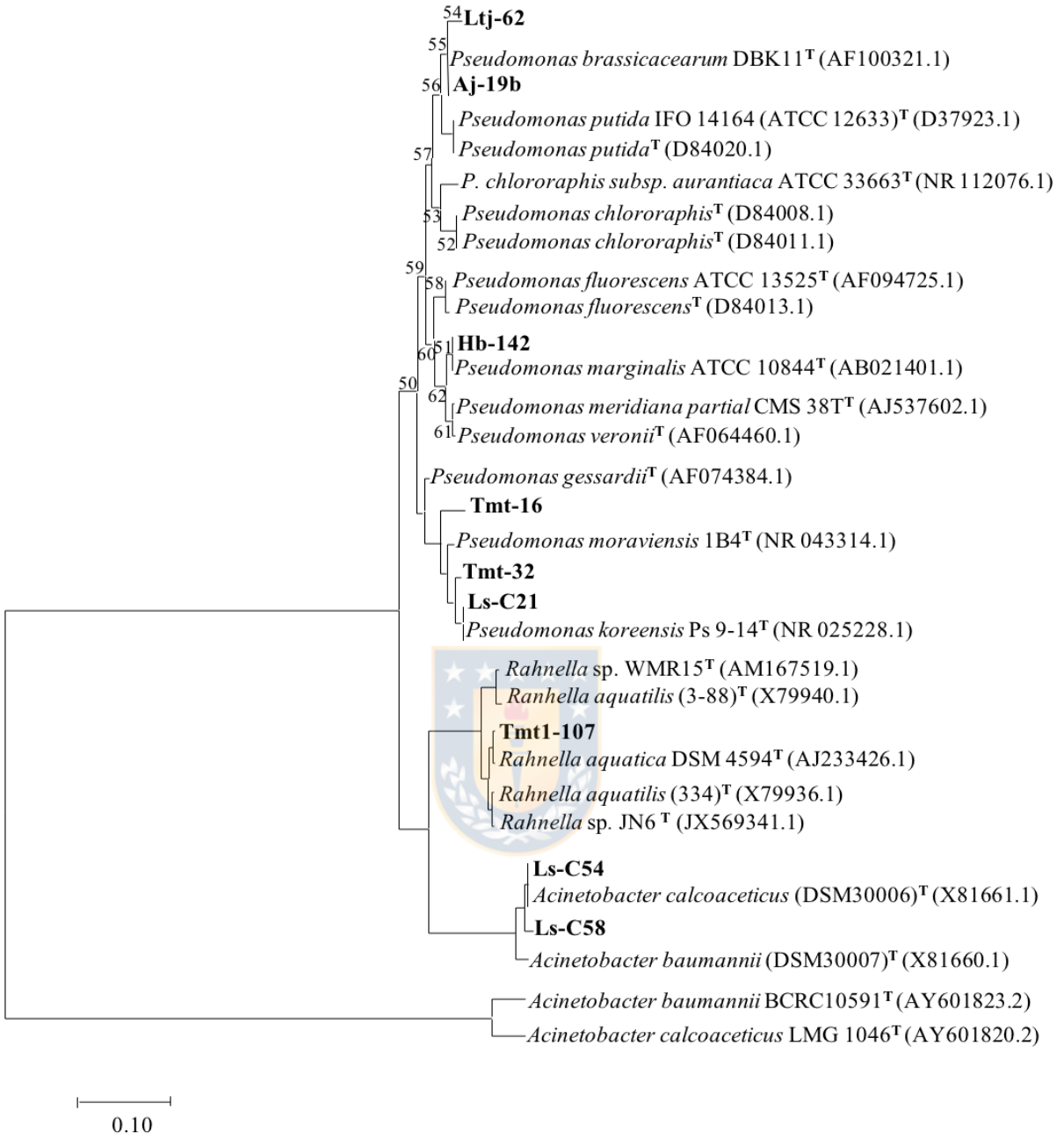
2014). In fact, *Pseudomonas* is a widespread bacterial genus, which presents the highest P solubilizing activity in rhizosphere soil (Bahena et al., 2015). *Pseudomonas azotoformans* has also been reported as K solubilizing bacteria (Saha et al., 2016).

LsC-58 and Ls-C54 were identified as belonging to the *Acinetobacter* genus and were closely related to the type strain *A. calcoaceticus* DSM30006^T (X81661.1), with similarity scores of 99.989 % and 100 %, respectively. *Acinetobacter calcoaceticus* has also been reported as PSB, and a Zn oxidizer (Rokhbakhsh-Zamin et al., 2011). *Acinetobacter* spp. has been described to have a high biological N fixation in sugarcane (Taulé et al., 2012). When associated with mycorrhizal fungi, this species has shown potential to contribute to remediating alkaline-saline soils contaminated with petroleum (Xun et al., 2015).

Tmt1-107 was identified as *Rahnella* sp., and as closely related to the type strain *R. aquatilis* 334T (X79936.1). *Rahnella* sp. is a bacterium described as PGPR in major agricultural crops. *Rahnella aquatilis* has been described as a PSB in the rhizosphere of *Eucalyptus* (Angulo et al., 2014). Furthermore, *Rahnella* sp., BIHB 783, has been described as an ammonia producing bacteria, and a P solubilizer, in barley, chickpea, pea, and maize (Vyas et al., 2010).

3.4.5 Endophytic capacity of the selected bacteria

Endophytic bacteria colonize any region within the epidermis of the plant root, and they can reside in apoplastic intercellular spaces and the xylem vessel apoplast. These bacteria establish in less competitive niches that present better conditions for N fixation and assimilation of fixed N by the plant (Carvalho et al., 2014). Of the plants inoculated in the pot trials, 30 different strains were isolated based on the physical appearance of each colony. However, when the genetic fingerprint of these isolates was determined, results did not coincide with the band patterns of the original strain. Therefore, none of the eight strains selected as NFB, PSB and KSB had the ability to act as endophytes (Figure 5).



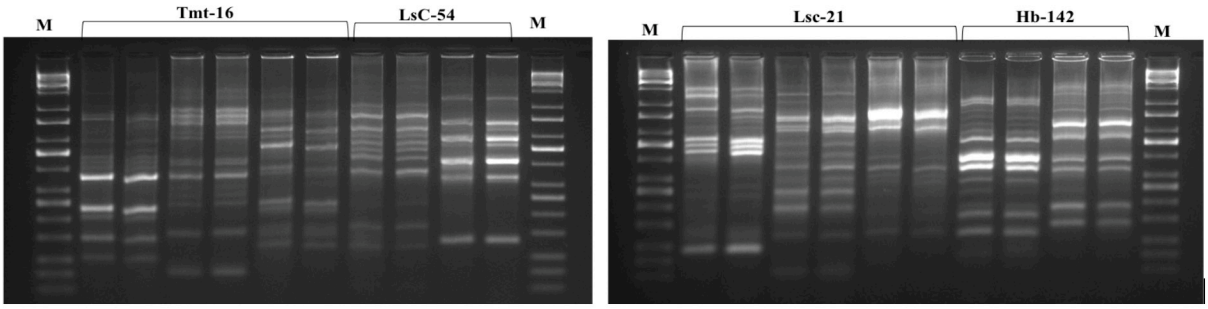
Fuente: Elaboración propia.

Figure 4. Neighbor joining phylogenetic tree based on 16S rRNA sequencing of phylogenetic tree of *Pseudomonas* spp., *Rahnella* spp. and *Acinetobacter* spp. Bootstrap values are indicated on branches only when higher than 70. The type strains sequences in the phylogram were obtained from GenBank (accession number in parentheses). The bacterial strains studied are marked in bold.

3.4.6 Effect of the selected bacteria in plant and soil

Effect of NFB. Tmt1-107 *Rahnella aquatica*, and Hb-142 *P. marginalis* did not generate a positive effect on tomato plant growth. On the other hand, Tmt-16, *P. gessardi* (7.38 cm), increased root growth in 23.57 % compared to the negative control (5.64 cm) ($p = 0.05$). However, Tmt-16 was the strain that resulted in the lowest plant height, reaching 5.50 cm ($p = 0.0047$), which was 2.17 cm lower than the control (7.67 cm). The results on higher root growth coincide with those of Naiman et al. (2009). The former reported an increase in root growth from 18 % to 26 % in tomato plants inoculated with *Bacillus subtilis* BEB-13bs (post-transplant) compared to the untreated control; the latter reported that there was a 40 % increase in root biomass in wheat inoculated with *P. fluorescens*.

Tmt-16, P. gessardi, significantly increased N content in plant tissue in 2.60 %, compared to the control and to strains Tmt1-107, Hb-142 ($p = 0.0013$). This strain maintained the highest amount of N available in the soil, 2.95 mg kg-1 ($p = 0.0006$), and a higher content of N-NH4+ 1.95 mg kg-1 ($p = 0.0296$) (Table 2). This indicates that Tmt-16, P. gessardi, is a N-fixing bacteria with potential use as biofertilizer or growth promoter. The change in root growth by Tmt-16, P. gessardi, resulted in greater root surface area for the uptake of nutrients and production, which also explains the higher N concentration in plant tissue. Studies in tomato indicate that inoculation with *Bacillus amyloliquefaciens* IN937a and *B. pumilus* T4 may increase plant growth and N availability. However, the effect of bacteria can vary and is influenced by factors such as N source, N rate, and soil fertility (Fan et al., 2017). In 2005, studies with the tomato 'Amalia' showed that applications of *Azospirillum* sp. (AzoFert) combined with 120 kg N increased yield in 11 % in comparison to the control, resulting in a reduction of 30 kg N ha^{-1} in the crop, which represented a decrease of 20 % N application (Alfonso et al., 2005).



From left to right, Tmt-16; band 1 and 2, control-strain, band 3 and 4 isolated from the leaf, band 5 and 6 isolated from the stem. LsC-54; band 1 and 2 control-strain, band 3 and 4 isolated from the leaf. LsC-21; band 1 and 2 control-strain, band 3 and 4 isolated from the leaf, band 5 and 6 isolated from the stem. Fuente: Elaboración propia.

Figure 5. PCR amplification of the bacterial isolates from leaf and stem of the plants inoculated.

In cereals, the estimates of biological N_2 fixation by free-living bacteria generally range between 5 and 50 kg N ha^{-1} yr^{-1} in enriched soil environments and plant residues (Roper and Gupta, 2016). The process of N_2 fixation varies between different bacterial genera. Most of biological N fixation is carried out by the activity of molybdenum nitrogenase, which is found in all diazotrophs (Carvalho et al., 2014). Of the large number of free-living N_2-fixing bacteria, the following genera have shown potential as biofertilizers or growth promoters: *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Derxia*, *Pseudomonas*, *Herbaspirillum*, *Burkholderia*, *Gluconacetobacter*, *Derxia*, *Beijerinckia* and *Azotobacter*, *Enterobacter*, *Bacillus*, *Alcaligenes*, *Klebsiella*, *Lysobacter* sp. and *Paenibacillus polymyxa* (Majeed et al., 2018). Of all these genera, only the genus *Pseudomonas* was identified in this study.

Table 2. Effect of the N-fixing bacteria on N content in plant and N availability in the soil.

Isolates	N uptake	N available in soil	$N\text{-}NH_4^+$ in soil	$N\text{-}NO^-_3$ in soil
	%	mg·kg^{-1}		
Tmt-16	2.60a	2.95a	1.95a	1.0
Tmt1-107	1.19c	1.80b	1.25b	0.55
Hb-142	1.25c	1.70b	0.90b	0.90
Control	1.73b	1.50b	0.95b	0.55

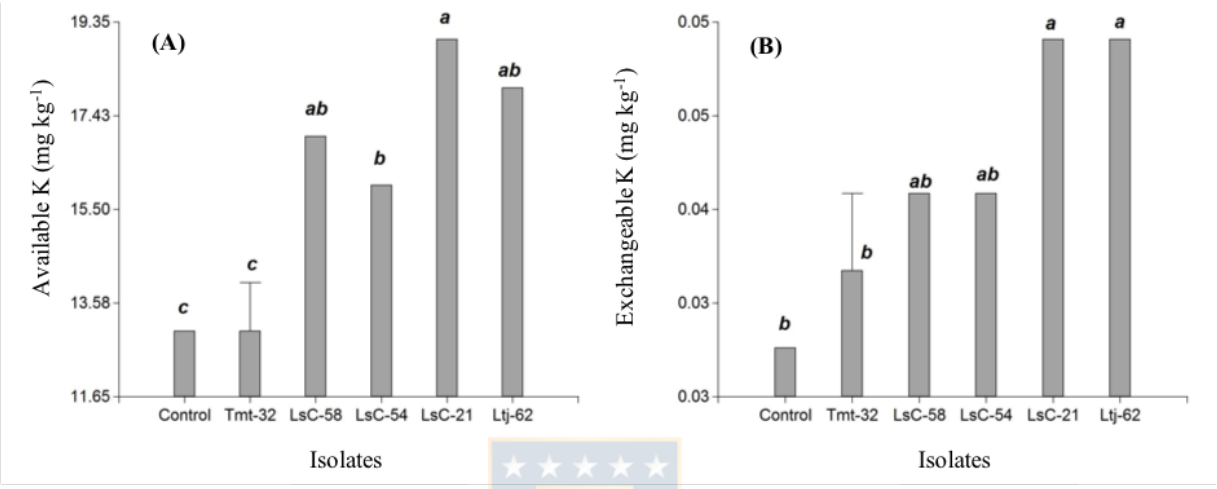
Mean values with the same letter are not significantly different ($p > 0.05$). Fuente: Elaboración propia.

Effect of PSB. Hb-142 *P. marginalis*, LsC-58 *A. calcoaceticus* and Tmt1-107 *R. aquatilis* did not generate a positive effect on tomato plant growth. Regarding P solubilization, these strains did not generate an increase in the initial content of available P in the soil (19.2 mg kg^{-1}). On the contrary, available P diminished, ranging from 14.8 to 16.8 mg kg^{-1}. P content in the plant tissue ranged between 0.13 % and 0.14 % and was similar to that of the plants without bacterial inoculum (0.14 %).

The effectiveness of PSB depends on the ability of the isolates to colonize the rhizosphere and maintain their biological activity, and on the types of metabolites produced and their release rate (Zhu et al., 2011). The performance of the PSB is influenced by with the nutritional richness and soil physicochemical properties such as organic matter, temperature and soil pH (Alori et al., 2017). The results obtained indicate that the effect of the bacteria on plants and soil was strongly influenced by the soil pH (5.66). This is due to the fact that tomatoes grow best in slightly acid soil with a pH range from 6.0 to 7.5, and for the best replenishment of P in the soil, the pH should be in the range of 6 to 7. Therefore, the increase in soil acidity from 5.66 to 5.3 at the end of the experiment probably resulted in the formation of aluminum and iron phosphates, which led to symptoms of P deficiency in plants. On the other hand, most solubilizing microorganisms can solubilize calcium phosphate complexes and only some can solubilize aluminum or iron phosphate. Therefore, further studies are required to evaluate these PSB, which were selected in laboratory, in another type of soil and/or with slightly acid pH ranges.

Effect of KSB. The five bacterial strains classified as KSB neither promoted growth in tomato plants nor increased K content. The K solubilizing strains Ls-C21 *P. koreensis*, Ltj- 62 *P. brassicacearum* and LsC-58 *A. calcoaceticus* reached 17.0 to 19.0 mg kg^{-1} available K ($p = 0.0002$) and 0.04 to 0.05 mg kg^{-1} of interchangeable K ($p = 0.0023$). Ls-C21, had the highest amount of available K 19.00 mg kg^{-1}, maintaining 6 mg kg^{-1} more than the soil without bacterial inoculation (Figure 6A, 6B). Sirajuddin et al. (2016) found that *B. pumilus* increases the solubility of K^+ and the antioxidant activity induced by tomato plants, which helps plants maintain their yields under stress conditions. About 90 % of K exists in forms of insoluble rock and silicate minerals, but soluble potassium is generally very low in the soil (Parmar and Sindhu, 2013). In fact,

K deficiency has become a major limitation for crop production. Inadequate K nutrition negatively affects root development, seed production and yield (Zhang and Kong, 2014). These results indicate that these three strains have potential as K solubilizers and can be an alternative to maintain potassium levels in the soil.



Means with the same letter are not significantly different ($p > 0.05$). Fuente: Elaboración propia.

Figure 6. Effect of K solubilizing bacteria on the soil: available K (a) and interchangeable K (b).

3.5. CONCLUSIONS

Four bacteria strains were selected for their capacity to fix N_2, three for their ability to solubilize P, and six for their capacity to solubilize biotite and K-feldspar, isolated from tomato, lentil, chili pepper, faba bean and lettuce crops in Andisol and Alfisol soils.

These bacteria selected in laboratory, four bacteria were selected because of their capacity to maintain levels of N and K available to plants, in an Ultisol soil with a low content of available N and K. Three of them belong to the genus *Pseudomonas*: Tmt-16, *P. gessardi*; Ls-C21, *P. koreensis*; Ltj- 62 *P. brassicacearum* and one corresponds to genus *Acinetobacter*: LsC-58, *A. calcoaceticus*, collected from an Ultisol and Andisol from tomato, lettuce and lentil crops. Tmt-16 increased root growth, which is a quality parameter in the production of tomato seedlings. P solubilizing strains did not show a positive effect on tomato plant growth or increase in available soil P.

These four endemic rhizobacteria of Chile can be potentially used as biofertilizers, allowing a reduction in the use of chemical fertilizers and a more sustainable production of tomatoes.

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IV. CAPITULO 4: NPK BIOAVAILABILITY AND GROWTH PROMOTION IN TOMATO INOCULATED WITH RHIZOBACTERIA UNDER DIFFERENT FERTILIZATION REGIMES

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4.1 ABSTRACT

Rhizobacteria are soil bacteria that can colonize the rhizosphere and improve nutrient availability through various mechanisms. The objective of this study was to evaluate NPK bioavailability and plant growth promotion in tomato inoculated with diverse rhizobacteria at different levels of fertilization. Three experiments were conducted under a factorial arrangement. In the N_2 availability experiment, the inoculation with Tmt-16 at 100% N increased available N in the soil by 44%. The inoculation with Hb-142+50%N and Tmt-16+0%N increased the N content on plant tissue from 20 to 71%, and Hb-142+50%N and Tmt1-107+50N% also increased dry matter content (25-29%). In the phosphorus solubilization experiment, there was no positive effect on P bioavailability or plant growth with bacterial inoculation. In the potassium solubilization experiment, the treatments Ltj-62+100%K, LsC-54+50%K and LsC-21+0%K increased levels of exchangeable and available K in the soil from 14 to 60%. The treatments did not increase K content in the plant tissue or improve plant growth. The various effects of the interaction between N_2 fixing, P solubilizing and K solubilizing bacteria with different fertilization levels offer a wide range of possibilities for the use of these

rhizobacteria in soils with different fertility levels, also allowing for sustainable tomato production.

Key words: exchangeable K, phosphorus and potassium solubilization, N_2 fixation, Dry matter.

4.2 INTRODUCTION

The increase in production and use of fertilizers in the last 20 years has not been reflected in increased crop yields. This could be due to the low efficiency in the use of nutrients, which in turn leads to greater environmental risk (Gouda et al., 2018). The rhizosphere, which is a zone of soil immediately surrounding a plant root, is a dynamic region governed by complex interactions between plants and microorganisms that are in close association with the root. The greater amount of soluble macro and micronutrients in the proximity of the soil-root interface has a positive effect on plant nutrition (Hayat et al., 2010). Therefore, the study and management of the interactions occuring in the rhizosphere improve nutrient use efficiency and crop productivity (Gouda et al., 2018).

Plant growth promoting rhizobacteria (PGPR) are soil bacteria that can colonize the rhizosphere and improve nutrient availability through mechanisms such as biological nitrogen fixation, phosphate solubilization, potassium solubilization, iron-chelanting siderophore production and organic matter mineralization (Bashan et al., 2014; Pii et al., 2015). PGPR inoculation in crops of agronomic interest have resulted in an increase in nitrogen and phosphorus, and other minerals that are made available to the plant (Meena et al., 2014).

According to the Office of Agrarian Studies and Policies (ODEPA), tomato is one of the major agricultural crops in Chile, with an estimated production of 388,000 tons for fresh consumption. Several studies on the effect of PGPR in tomato have reported increased levels of N, P, K and B in the plant, as well as positive effects on growth and germination, yield, fruit quality and stress tolerance (Ordookhani et al., 2010; Sirajuddin et al., 2016; Mehta et al., 2015; Majeed et al., 2018).

PGPR can be classified as biofertilizers when they act as a plant nutrition source and enrichment source to replenish or rebuild the nutrient cycle between the soil, plant roots and microorganisms present (Vejan et al., 2016). Biofertilizers are becoming more frequently available in the market of agricultural inputs in Chile. However, the Sub-department of Organic Agriculture of the Chilean Government's Agricultural and Livestock Service (SAG) has reported that 80 % of these are imported. Imported biofertilizers have not necessarily been previously tested in the crops in which they are actually being applied, and are not necessarily adapted to the climatic conditions of Chile. Therefore, there is a need for specific technologies that can meet the requirements of each region of Chile.

Several PGPR have been isolated from different soils and crops in Central Chile, showing that they can promote root and aerial growth, and increase nodule formation in legumes (Gerding et al., 2017; Sepúlveda-Caamaño et al., 2018; Cedeño-Garcia et al., 2018). The objective of this study was to evaluate NPK bioavailability and plant growth promotion in tomato inoculated with diverse rhizobacteria at different levels of fertilization.

4.3 MATERIALS AND METHODS

NPK bioavailability and growth promotion of 'Cal Ace' tomato plants were evaluated based on the effect of the interaction between rhizobacteria and fertilization levels. The rhizobacteria strains were selected from *in vitro* experiments for their ability to fix N and solubilize P and K (Reyes-Castillo et al., 2019). Plants were grown in speedlings using soil of the following characteristics as a substrate: 7.0 mg kg^{-1} available N (nitrates N-NO_3 4.90 mg kg^{-1} and ammonium N-NH_4^+ 2.10 mg kg^{-1}), 19.20 mg kg^{-1} available P, 40.90 mg kg^{-1} available K, 5.66 pH in water and 4.18 % organic matter. The soil was classified as Isotic, mesic Dystric Xeropsamments belonging to the Arenales series.

Three experiments were conducted under a factorial arrangement with a randomized experimental block design in order to evaluate: 1) Nitrogen availability, consisting of 12 treatments (4X3), and including 3 N_2 fixing bacteria (NFB): Tmt1-107,

Tmt-16 and Hb-142, plus a negative control, under 3 different levels of nitrogen fertilization : 0, 50 and 100 % N; 2) Phosphorus solubilization, consisting of 8 treatments (4 X 2), and including 3 phosphorus solubilizing bacteria (PSB): LsC-58, Hb-142 and Tmt1-107 + 1 negative control, under 2 levels of phosphate fertilization: 50 and 100 % (% P); 3) Potassium solubilization, consisting of 18 treatments (6 X 3), and including 5 potassium solubilizing bacteria (KSB): Tmt-32, LsC-21, LsC-54, LsC-58 y Ltj-62 + 1 negative control, under 3 levels of potassium fertilization: 0, 50 and 100 % (% K). Each treatment consisted of three replicates, using 27 plants as an experimental unit.

4.3.1 Fertilization levels

Fertilization doses for the production of tomato seedlings were calculated based on crop demand and soil supply. The demand for NPK was 2.77, 0.41 and 4.18 kg t^{-1}, with an estimated yield of 100 t ha^{-1}, and requirements of 35 % N, 30 % P and 20 % K for this phenological stage. Fertilization doses for each experiment were prepared with analytical grade reagents (stock solutions) and distilled water (Table 3). The solutions were then autoclaved at 121 °C at 15 psi for 20 minutes. Stock solutions were refrigerated.

The experiments were carried out in a phytotron with high-pressure sodium steam lamps (400 W Gro-lux®, Osram Sylvania Ltd., Danvers, MA, USA), providing a minimum photosynthetic photon flux density (PPFD) of 400-500 mol $m^{2} \cdot s^{-1}$. The temperature ranged from 21 to 27 ° C during germination and from 21 to 24 ° C during vegetative growth, with a relative humidity between 60 % and 80 %.

Seeds, soil and germination trays were disinfected prior to sowing. The seeds were disinfected with 5 % hypochlorite (w/v) for 3 minutes and with 70 % ethanol (w/v) for 1 minute, followed by six washes in sterile distilled water. The soil was disinfected with steam at an initial temperature of 100° C and then maintained at 82° C for 30 minutes. The germination trays were disinfected with 10 % sodium hypochlorite (w/v).

Table 3. Stock solutions per fertilization level for each experiment, dose per tray, 3.645 kg soil.

Analytical reagent	[z]Fertilization levels							
	N_2 fixation			Phosphoru solubilization		Potassium solubilization		
	100%	50%	0%	100%	50%	100%	50%	0%
KNO_3	2.407	2.407	0.000	2.407	2.407	2.407	1.203	
$NH_4H_2PO_4$	0.520	0.520	0.520	0.520		0.520	0.520	0.520
K_2SO_4			4.148					
$(NH_4)_2SO_4$	8.152	0.347	0.000	8.152	2.288	8.152	1.379	1.582
Mg $(NO_3)_2$	2.288			2.288		2.288		
$MgSO_4$ $7H_2O$	2.560			2.560		2.560		
Ca $(NO_3)_2$	1.338	1.338	1.338	1.338	2.560	1.338	1.338	1.338
$MnSO_4$ H_2O	0.008			0.008		0.008		
$ZnSO4$ $7H_2O$	0.006	0.008	0.008	0.006		0.006	0.008	0.008
$CuSO_4$		0.006	0.006		0.008		0.006	0.006
H_3BO_3	0.011			0.011	0.006	0.011		

Fuente: Elaboración propia.

4.3.2 Plant inoculation

Strains were grown in different culture media depending on the type of bacteria. Nitrogen fixing bacteria (NFB) were grown on Burk's nitrogen-free medium (Hartono et al., 2016); Phosphorus solubilizing bacteria (PSB) were grown on Pikovskaya medium (Hariprasad and Niranjana, 2009); and potassium solubilizing bacteria (KSB) were grown using modified Aleksandrov medium (Meena et al., 2015). Liquid media were inoculated with each isolate and incubated in a rotary shaker (150 rpm) at 25 °C for 3 days for NFB and PSB, and at 28±1°C for 7 days for KSB.

Bacterial concentration for each strain was standardized at 10^7 CFU mL^{-1} in 1.0 % sucrose (v/v) determined through absorbance (λ= 600 nm). For inoculation, 1 mL of standardized bacterial suspension was placed aseptically in the root zone of each plant. Plants were inoculated twice: at emergence stage and 15 days after emergence.

4.3.3 Plant growth promotion

Root dry matter, plant tissue dry matter and total dry matter (g) were measured 35 days after sowing. The seedlings were removed from the soil; then the root shoots were divided and carefully washed. Subsequently, the samples were dried in an oven at 70 ° C, and dry weight was measured after the roots and shoots reached their constant weight.

4.3.4 NPK determination in plant tissue and soil

NPK levels were determined through soil analysis methods of the Commission for Standardization and Accreditation of the Chilean Society of Soil Science. Plant N analysis was carried out by the digestion method and colorimetric determination; inorganic N in soil was determined by colorimetry, nitrates via nitration of salicylic acid, and ammonium using Nessler reagent. Phosphorous content was determined by calcination and colorimetric determination of the phospho-vanadomolybdate, and K content was determined through calcination and atomic emission spectrometry.

4.3.5 Compatibility between bacterial strains

At the end of the experiment, compatibility between the strains that promoted higher plant growth and greater NPK bioavailability was evaluated by the disc diffusion method in agar medium with modifications (Balouiri et al. 2016). The evaluation was done using the following index: 0 = no bacterial growth, 1= inhibition area > 5 mm, 2= inhibition area < 5 mm, and 3 = no inhibition.

4.3.6 Statistical analysis

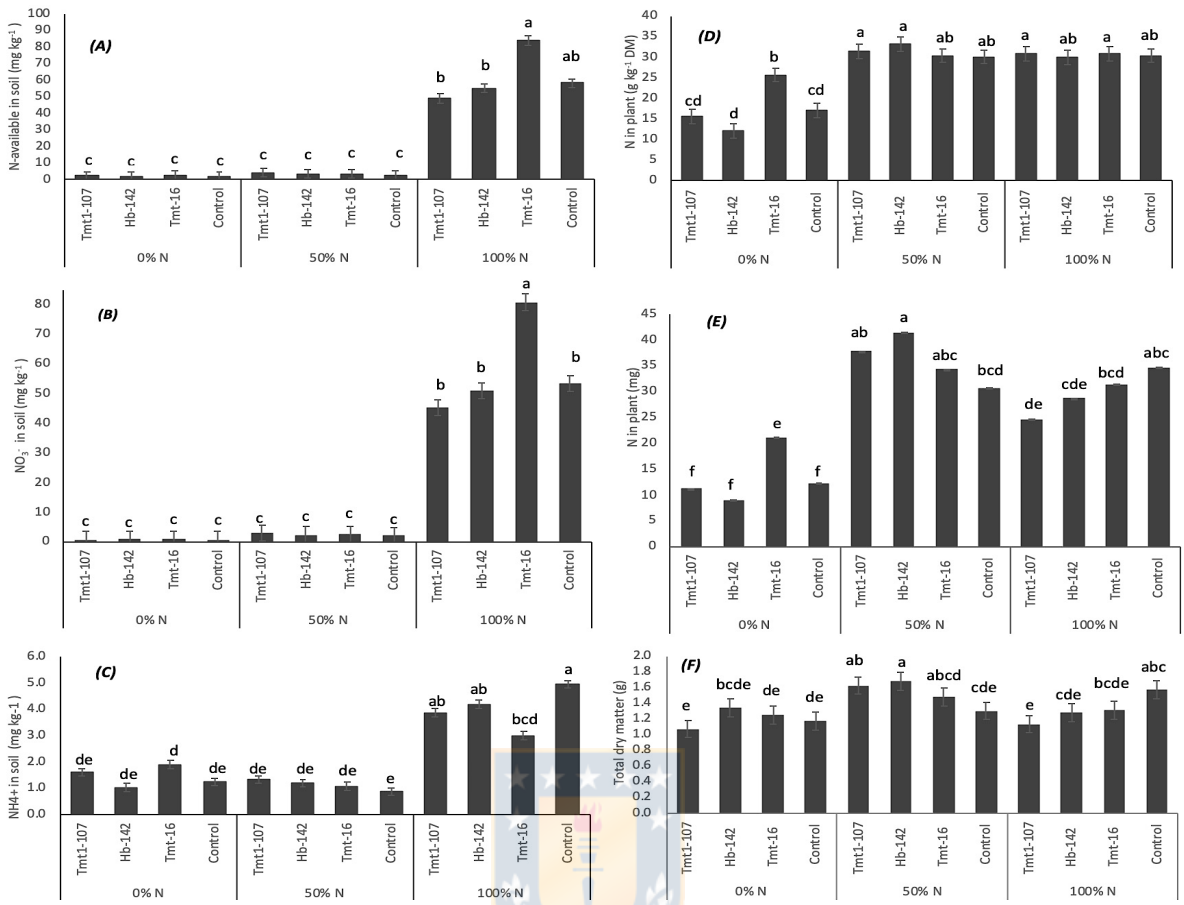
The data obtained from each experiment (trials in a randomized block design with three replicates) were subjected to an ANOVA and a means comparison test (Fisher's LSD), with a significance level of $p \leq 0.05$ using the InfoStat / E 2013 software.

4.4 RESULTS AND DISCUSSION

4.4.1 N availability experiment

Effect on N availability in the soil. Differences in soil N availability were observed between the different inoculation and fertilization treatments (Figure 7A). The initial available N content in the soil was 7.0 mg kg^{-1}. At the end of the experiment, values decreased in the treatments NFB+0%N and NFB+50%N with values ranging from 1.80 to 2.73 mg kg^{-1} and 2.90 to 4.10 mg kg^{-1}, respectively. At the fertilization levels of 0 and 50%N there was no significant effect of bacterial inoculation, while in the 100% N treatments, levels of available N varied between 49.03 and 83.83 mg kg^{-1}; and soil inoculated with Tmt-16+100%N recorded the highest content of available N in the soil (83.83 mg kg^{-1}), which corresponds to 43.82 % more than the Control+100%N (Figure 7A).

Estimates of N_2 biological fixation by free-living bacteria range between 5 and 50 kg N ha^{-1} $year^{-1}$, varying according to the environmental conditions of the soil and the type of organism (Roper and Gupta, 2016). A study in wheat and sorghum showed that inoculation with *Azospirillum brasilense*, increased available N by up to 58 % (Carvalho et al., 2014). Inoculation of tomato plants with *Bacillus* spp. increased N availability. However, the effect of bacteria varied and was influenced by factors such as N source, N rate and soil fertility (Fan et al., 2017). In this work, the variation in NO_3- content accounted for the differences in available N in the soil (Figure 7B), without correlating with the amount of $NH4^+$ in the soil (Figure 7C). It should be noted that Tmt16+0%N generated a concentration of NH_4^+ in the soil higher than the treatments with 50 % N fertilization, including the control. Same as for the N available values obtained, no differences were found between the treatments 0 % N and 50 % N in terms of NO_3^- content. However, in NFB+100%N, the treatment Tmt-16+100%N (80.87 mg kg^{-1}) generated 51.6 % more NO_3^- than the control (53.34 mg kg^{-1}) (Figure 7B). NO_3^- can be readily absorbed by plants and does not need any previous transformation (Liu et al., 2017; Marschner, 2012).



Fuente: Elaboración propia.

Figure 7. Effect of the inoculation with N_2 fixing bacteria (NFB) and different levels of nitrogen fertilization in the soil: (A) available N, (B) NO_3^-, (C) NH_4^+; and in the plant: (D) N concentration based on dry matter, (E) N content and (F) total dry matter production. Values are arithmetic means ± SE of n = 3 replicates.

Effect on N nutrition in the plant. N is part of the organic compounds in the plant. In fact, it is a major component of amino acids and enzymes, and makes possible processes of ionic absorption, photosynthesis, respiration, synthesis, cell reproduction and differentiation, inheritance and throughout the plant's metabolism (Pii et al., 2015). Nutrient uptake and accumulation rate in the plant increase in the form of a saturation curve as its availability increases in the nutrient medium (Marschner, 2012).

N sufficiency in the plant ranges from 20 to 50 g kg^{-1} DM (dry matter); values lower than 20 g kg^{-1} DM generate a critical N level in the plant. In the treatments NFB+50%N

and NFB+100%N, N concentration in the plant tissue was within the sufficiency range, with values that varied from 29.9 a 33.2 g kg^{-1} DM (Figure 7D).The treatments Hb-142+50%N (32.2 g kg^{-1} DM), Tmt1-107+50%N (31.5 g kg^{-1} DM) and Tmt-16+50%N (30.04 g kg^{-1} DM) recorded values that were similar to the Control+100%N (30.4 g kg^{-1} DM), but with differences in available N in the soil: low level for the treatments (ranging from 3.6 from 4.1 mg kg^{-}) and high level for the Control (58.29 mg kg^{-1}) (Figure 7A and 7D). Therefore, these results indicate that inoculation with strains Hb-142, Tmt1-107 and Tmt-16 in a soil with a low level of available N would help improve N concentration in the plant.

In the treatments with 0%N, N levels in the plant were critical, except for the treatment inoculated with Tmt-16 which generated an N concentration of 25.7 g kg^{-1} DM in the seedlings, which corresponds to 50.3 % more N in plant tissue than its respective control (Figure 7D). This results are promising, in comparison to what has been obtained by Mehta et al. (2015) in studies in tomato inoculated with *Bacillus circulans*, where N in plant tissue was increased by 18.75 %, and Yachana et al. (2013) in studies in rice inoculated with *Bacillus pumilus* and *Pseudomonas pseudoalcaligenes*, increasing N concentrations in plant tissue by 26 %.

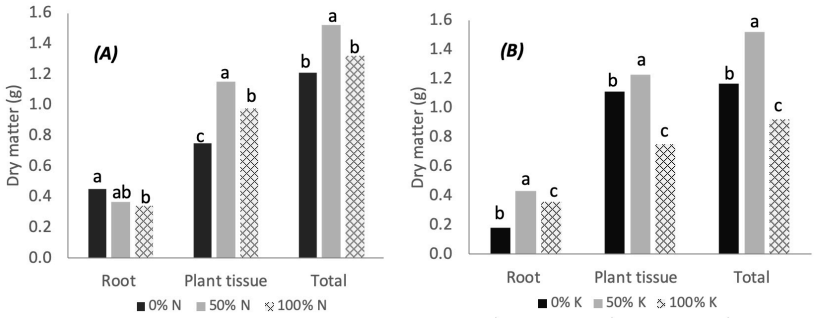
Clearer differences were found between treatments when plant tissue N was calculated based on dry matter production. The NFB treatments under different N fertilization rates resulted in an increase in N content in the plant tissue when the fertilization dose increased from 0 to 50 % but decreased when the fertilization dose increased from 50 to 100 %. However, the three NFBs had different behavior within each level of fertilization (Figure 7E). On the contrary, N content in the plant tissue of the control treatments increased according to the level of fertilization. The highest N content in the plant was obtained with Hb-142+50% (41.5 mg), which generated 19.75 % more N than the Control+100% (34.65 mg). Tmt-16+0%N was the treatment that recorded the highest N content (21.074mg) of those with 0 % N, producing 71.16 % more N than its respective control without bacterial inoculation (Figure 7E).

Effect on dry matter production. Differences in the production between root dry matter and aerial dry matter were defined by the level of fertilization (% N). The highest

production of aerial dry matter was recorded with 50 % N fertilization. On the contrary, the highest root dry matter production was recorded with 0 % N fertilization (Figure 8A). These differences between root and aerial dry matter suggest that there is an interaction between inoculation treatment and fertilization levels in terms of total dry matter (Figure 7F).

Inoculation with Hb-142+50%N (1.68 g) and Tmt1-107+50N% (1.62 g) recorded the highest total dry matter, producing 29.23 and 24.62 % more than the Control+50%N (1.30 g); However, these treatments were unable to promote growth in a soil with a 100% N fertilization (Figure 7F).

Similar results were obtained in studies in tomato conducted by Alfonso and Leyva (2006), who inoculated plants with *Glomus clarum and Azospirillum brasilense* + chemical N fertilization and reported a greater production of dry matter 31 days after inoculation; and by Mehta et al. (2015), who inoculated *Bacillus circulans* CB7 and reported a 52.9 % increase in shoot dry matter and 31.4 % increase in root dry matter. In addition, another study in tomato showed that inoculation with *Bacillus amyloliquefaciens* IN937a and *B. pumilus* T4 can increase plant growth.



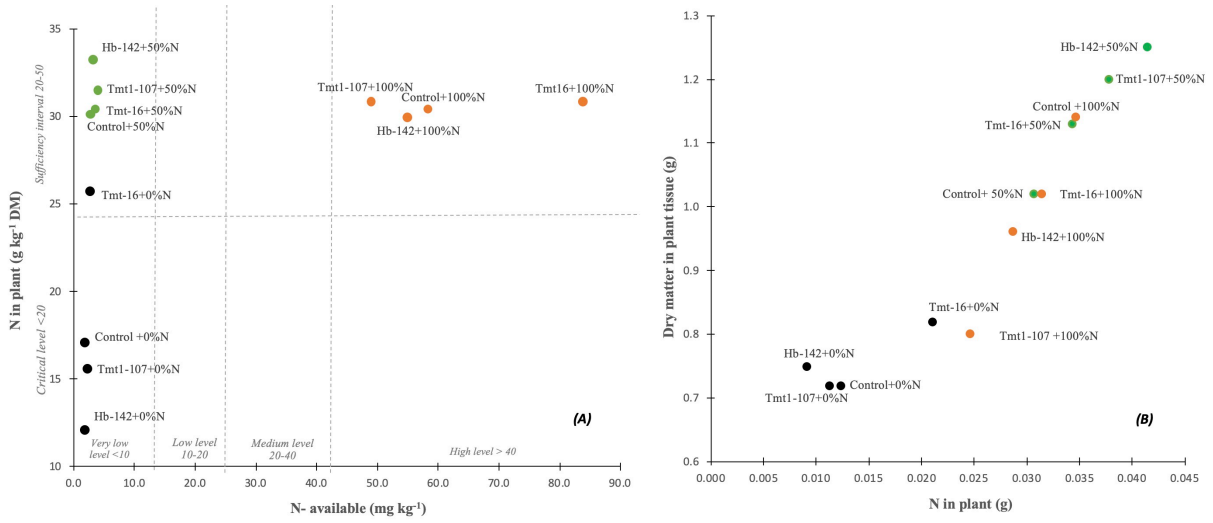
Fuente: Elaboración propia.

Figure 8. Effect of fertilization levels on dry matter production of tomato seedlings: root, aerial and total; (A) N availability experiment and (B) K solubilization experiment. Values are arithmetic means ± SE of n = 3 replicates.

Effect on N bioavailability and growth promotion. There are several factors involved in the efficiency of N biological fixation process: nitrogen fixing bacteria should

be able to compete with soil bacteria; the content of available N should be low, in particular the amount of NH_4^+ as this can be a repressor of nitrogenase synthesis; soil pH should be close to neutrality; also aeration and an adequate content of molybdenum (Mb), calcium (Ca) and phosphorus (P) are required (Nuñez et al., 2012). Based on the N bioavailability generated by the treatments, it can be stated that the strain Tmt-16 is an N_2 fixing bacterium. Tmt-16 behaved differently depending on the level of nitrogen fertilization. Regarding plant nutrition, Tmt-16+0%N generated the highest N concentration in plant tissue, and helped increase N content in the plant while still growing in a soil with low available N; this was not observed at 50 % and 100 % nitrogen fertilization (Figure 9). This indicates that the strain Tmt-16 could be inoculated in tomato seedlings grown in a soil with low available N, keeping N levels in the plant tissue within the sufficiency range but without increasing available N levels in the soil. Similar results were obtained in wheat plants grown in N-deficient media and inoculated with the *nifH* mutant of *Klebsiella pneumoniae*, showing severe signs of N deficiency in contrast to plants inoculated with the wild-type *K. pneumonia*, which showed no deficiency (Iniguez et al., 2004). In addition, the inoculation of a mixture of diazotrophic bacteria in sugarcane grown in soils with different levels of fertilization, resulted in a higher biological N fixation in the soil with low N content (Oliveira et al., 2003).

The strain Tmt-16+100%N helped increase NO_3^- content and available N in the soil, but did not increase N levels in the plant tissue. The low contributions by NFB observed in soils with a high N content may be partly explained by the effect of N on nitrogenase activity. In addition to this, there are discussions on whether death and subsequent mineralization of diazotrophic bacteria could indirectly release significant amounts of fixed N (Hoffman et al., 2014; Carvalho et al., 2014).



Fuente: Elaboración propia.

Figure 9. Effect of the inoculation with N_2 fixing bacteria (NFB) and different levels of nitrogen fertilization on the relationship between: (A) concentrations of available N in the soil and N in the plant, and (B) N content in the plant and production of dry matter in the plant tissue.

Some bacteria have proved to be useful for increasing N absorption in a low N soil due to the production of cytokinins and ACC deaminase activity, which favors leaf growth and a greater nitrogen investment in the leaves (Esquivel-Cote et al., 2010). The strains Hb-142 and Tmt-16 are registered as bacteria producing the enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACC) and strain Tmt1-107 as a bacterium producing indole-3-acetic acid (IAA) (Data from Microorganism Collection - FAUdC). These phytohormones help improve plant growth by promoting root growth, which indirectly helps increase N uptake (Sepúlveda-Caamaño et al., 2018; Cedeño-Garcia et al., 2018). The strains Tmt-16 *Pseudomonas gessardi* and Hb-142 *P. marginalis* belong to the genus *Pseudomonas* (Reyes-Castillo et al., 2019), which has been extensively described as PGPR of several plants and N_2 fixing bacteria in rice (*P. monteilii* y *P. mandelii)* (Haibibi et al. 2014). The strain Tmt1-107 belongs to the species *Rahnella aquatica* (Reyes-Castillo et al., 2019). This species has been described as PGPR because of its biocontrol capacity, N_2 fixing bacterium in tomato, apple, corn, wheat, barley, chickpea and *Pisum sativum* L. (Vyas et al., 2010).

4.4.2 Phosphorus solubilization experiment

The statistical analysis (ANOVA) showed that the interaction between the PSB (Hb-142 *P. marginalis*, LsC-58 *A. calcoaceticus* and Tmt1-107 *R. aquatica* and the 50 and 100 % P fertilization levels, was not significant in terms of P bioavailability, nor in growth promotion in the tomato seedlings (*Solanum lycopersicum* L.). Available P in soil varied from low to medium levels (14.93 to 20.47 mg kg^{-1} (SE= 0.98)); P values in the plant ranged from 1.3 to 1.5 g kg^{-1} DM (SE= 0.047), which fall below the P levels regarded as optimum in the plant (2.0 to 5.0 g kg^{-1} DM), while dry matter production reached values between 2.59 and 3.21 g (SE= 0.32).

Crops recover 10 to 30 % of the P applied to the soil. The rest, which is consumed by microorganisms, is precipitated in the form of insoluble compounds or is strongly absorbed by the colloidal complex of the soil. This form of P is not readily available to plants and has been called fixed P (Behera et al., 2014). The P fixation in the soil was 38.88 %, while available P was 40.90 mg kg^{-1}, with a moderately acidic pH of 5.66, and a high saturation of Al^{3+} 5.45 % and 28.8 mg kg de Fe^{3+}.

The effectiveness of PSB depends on the ability of the isolates to colonize the rhizosphere and maintain their biological activity, as well as on the types of metabolites produced and their release rate (Zhu et al., 2011).The performance of PSB is influenced by the nutritional status and physicochemical properties of the soil, such as organic matter, temperature and soil pH (Alori et al., 2017). For these bacteria to function or help solubilize P, higher soil P levels or soil with different mining and texture properties may probably be required.

ANOVA of P-fertilization was significant only in the variable soil available P ($P<0.0001$). This was higher under 100 % fertilization levels, with a mean value of 19.11 mg kg^{-1} P_2O_5; 3.59 mg kg^{-1} more than the 50 % fertilization level (15.52 mg kg^{-1}). Both treatments kept soil available P levels in the mid-range (11-20 mg kg^{-1}). The initial available P of this soil was de 19.2 mg kg^{-1}, so that 100 % fertilization corresponded to maintenance of normal levels, while 50 % was deficient. Bacterial inoculation was expected to help correct this maintenance fertilization and increase the levels of available P in the soil through mechanisms to produce inorganic/organic acids (pH

reduction) to release the phosphates and cations of Ca^{2+}, Fe^{2+} and Al^{2+} (Behera et al., 2014). However, no significant differences were found between the treatments. This indicates that not all PSB are suitable for all types of soil or under different fertilization rates. Therefore, further research is required to evaluate these PSB in other soil types and/or with slightly acidic pH ranges.

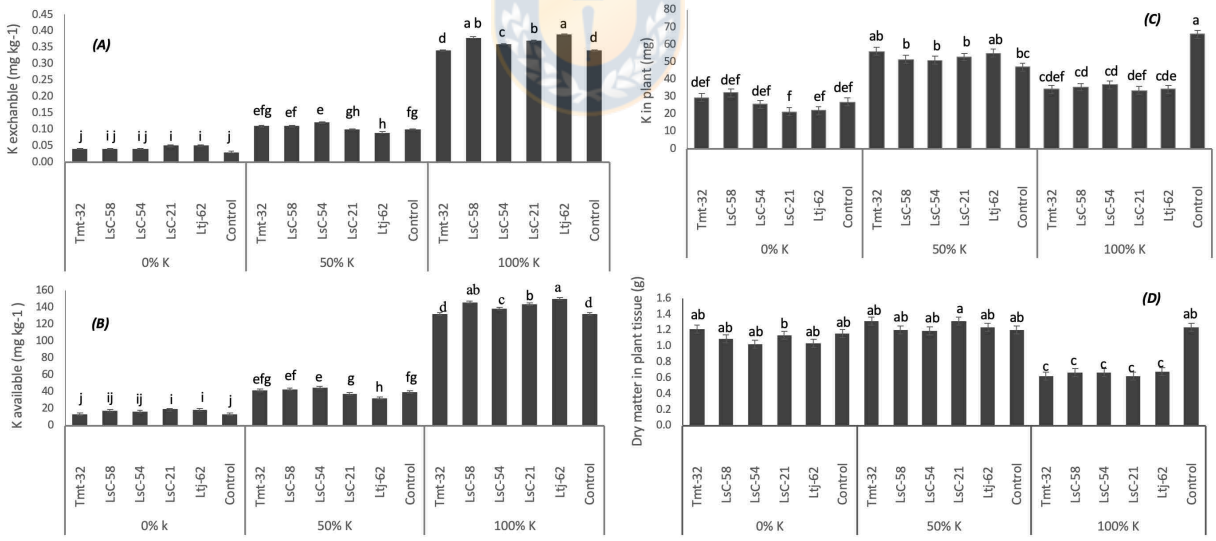
4.4.3 Potassium Solubilization experiment

Plants absorb K only from the soil. The amount of available K to the plants depends on the total K content (structural) and K dynamics in the soil. In the last decade, the level of available K in the soil has decreased due to the rapid development of agriculture and application of unbalanced fertilizers. Potassium solubilizing bacteria (KSB) can convert the insoluble or mineral structural K compounds into soluble forms in the soil, making them available to plants (Parmar and Sindhu, 2013; Meena et al., 2015).

Effect on K availability in the soil. K exists in four main forms in the soil: dissolved or solution K, exchangeable K, non-exchangeable K and structural K (Etesami et al., 2017). The results of exchangeable and available K had the same behavior pattern as a result of the interaction between KSB and fertilization levels. Release of non-exchangeable K to the exchangeable form occurs when levels of solution and exchangeable K are reduced due to crop uptake, leaching or increased microbial activity. As solution and exchangeable K decreases in the soil, non-exchangeable K reserves release K to the soil solution. Thus, exchangeable K is in equilibrium with the soil solution and it is easily released when plants absorb K from the soil solution (Kumar et al. 2013).

Silt, clay and sand are the largest reservoir of K. The most common deposits of K are feldspar and mica. Alluvial soils are rich in mica with enough non-exchangeable K. Non-exchangeable K (fixed K) is held between adjacent tetrahedral layers of dioctahedral and trioctahedral micas, and constitutes 1 to 10 % of the soil K (Wang et al., 2016). The soil used in this study is an alluvial soil with a K fixation capacity of 89.3 %, and low concentrations of exchangeable K (0.10 cmol kg^{-1}) and available K (40.9 mg kg^{-1}). Based on the results of exchangeable K ($P < 0.0001$), the treatments Ltj-

62+100%K (0.39 mg kg^{-1}) and LsC-58+100 K (0.38 mg kg^{-1}) recorded the highest exchangeable K rates, which corresponds to 15 % more than the Control+100%K (0.34 mg kg^{-1}) (Figure 10A). The treatment Ltj-62+0%K (0.05 mg kg^{-1}) produced 66.6 % more exchangeable K than the Control+0%K (0.03 mg kg^{-1}). This indicates that the Ltj-62 strain in interaction with 0 and 100 % potassium fertilization levels has the potential to increase exchangeable K. However, the Ltj-62 strain produced 10 % less than the Control with a 50 % K fertilization (Figure 10A). The treatment with the strain LsC-54+50%K produced the highest exchangeable K, which corresponds to 20 % more than the Control+50%K and 33.3 % more than the strain Ltj-62+50%K. Regarding the interactions of KSB+0%K, the treatments Ltj-62+0%K and LsC-21+0% K generated the same exchangeable K values in the soil, reaching 66 % more than the Control+0%K (Figure 10A). Species such as *Bacillus mucilaginosus*, AS1.153 and *Pseudomonas azotoformans* have shown that they can solubilize K from montmorillonite, kaolinite and K feldspar and biotite (Saha et al. 2016).



Fuente: Elaboración propia.

Figure 10. Effect of the interaction between the inoculation with K solubilizing bacteria (KSB) and potassium fertilization levels (% K): (A) exchangeable K and (B) available K in the soil, (C) K content in the plant and (D) dry matter in the plant tissue. Values are arithmetic means ± SE of n = 3 replicates.

The KSB+100%K treatments generated the greatest amount of available K in the soil, ($P<0.0001$). In particular, the treatments Ltj-62+100%K (149.8 mg kg^{-1}), LsC-58+100%K (145.8 mg kg^{-1}), LsC-21+100%K (142.9 mg kg^{-1}) and LsC-54+100%K (139.8 mg kg^{-1}) generated more available K in the soil than the Control+100%K (132.3 mg kg^{-1}) (Figure 10B). The interaction of strains Ltj-62, LsC-58, LsC-54 and LsC-21, under 0, 50 and 100 % K fertilization showed that these bacteria respond differently. The treatment Ltj-62+100% K generated 14 % more available K than the Control+100%K. The treatment LsC-54+50%K recorded 13.96 % more available K than the Control+50%K (39.4 mg kg^{-1}). At 0 % fertilization level, the strain LsC-21 achieved the highest available K (19 mg kg^{-1}), reaching 46 % more than its respective Control (13.0 mg kg^{-1}) (Figure 10B).

The results show that the interaction between KSB and fertilization levels is diverse since the same bacterium behaves differently depending on the K fertilization level (0, 50 and 100 % K). Data obtained in terms of exchangeable and available K (initial and final) shows that strains Ltj-62, LsC-58, LsC-54 and LsC-21 can solubilize K in soil with low available K content (Figure 10B). The main mechanism of mineral K solubilization is the secretion, by diverse rhizosphere microorganisms, of various types of organic acids (succinic, citric, gluconic, α-ketogluconic and oxalic), and inorganic acids (acetate, citrate and oxalate). When these are released into the rhizosphere cause the fixed K of minerals to be released and dissolved by processes of acidolysis, chelation and complexolysis (Meena et al., 2015; Meena et al., 2014). Sirajuddin (2016) found that inoculation with *Bacillus pumilus* increases K^+ solubility and the antioxidant activity induced by tomato plants, particularly in the superoxide dismutase (SOD) and catalase (CAT) enzymes when they are boron-stressed, helping tomato plants keep yields under this stress.

Effect on K nutrition in the plant. Potassium (K) is an activator or cofactor of more than 50 enzymes of carbohydrate and protein metabolism. It acts as the main cation to establish cell turgor pressure and maintain cell electroneutrality. K participates in osmotic processes, opening and closing of the stomata, photosynthesis, carbohydrate transport, respiration, synthesis and symbiotic N_2 fixation (Maathuis, 2009). K content in the plant tissue ($P=0.0022$) was higher in the treatments

Control+100%K, Tmt-32+50%K, and Ltj-62+50% K, with contents of 65.93, 56.01 and 54.9 mg, respectively (Figure 10C). Therefore, these strains (Tmt-32 and Ltj-62) managed to solubilize K and make it available to plants with only 50 % of the potassium fertilization required by the plant. Ordookhani et al., (2010) evaluated the effect of inoculation with PGPR (*Pseudomonas* + *Azotobacter* + *Azosprillum*) and arbuscular mycorrhizae (AMF) in tomato (*Lycopersicon esculentum* L. F1 hybrid, GS -15). The results indicate that the use of PGPR+AMF resulted in greater amounts of potassium, lycopene and fruit antioxidant activity. Etesami et al., (2017) indicated that K absorption by plants can be increased by using KSB as bioinoculants that further increase crop productivity. The inoculations with KSB at 50% K achieved a higher K content in the plant compared than with KSB at 100 and 0% K (Figure 10C), suggesting that high and low levels fertilization in the soil limit the activity by KSB.

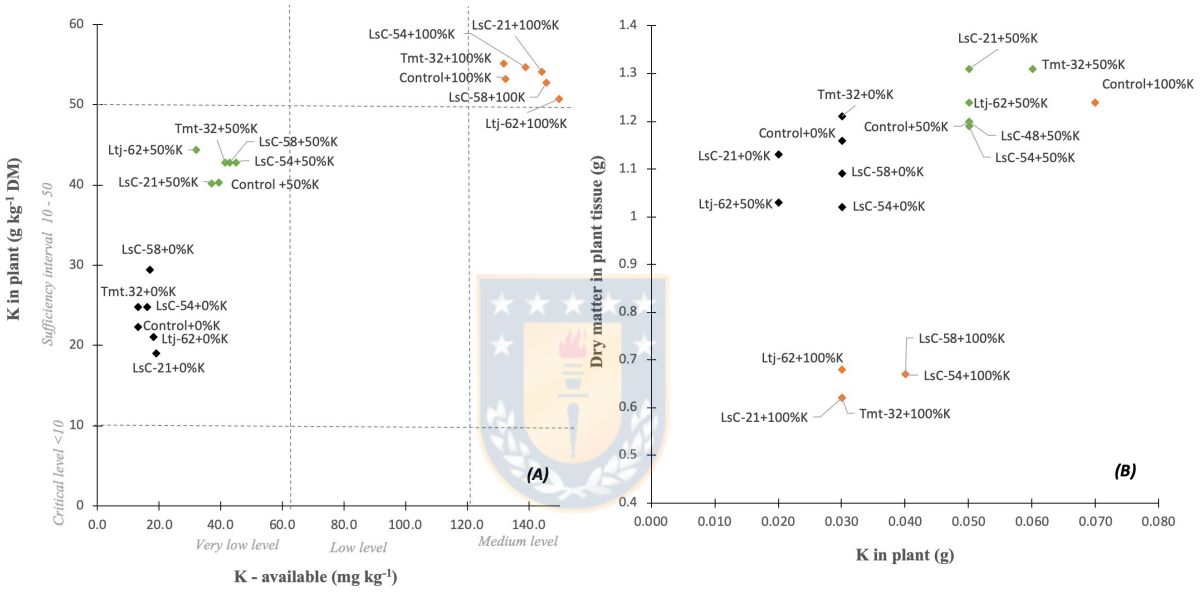
The Control+100%K produced the highest K content in the plant tissue, reaching 53.2 g kg^{-1} DM and being higher than levels considered as sufficient for the plant (10-50 g kg^{-1} DM). The treatments formed by KSB+100%K presented K concentrations higher than those required by the plant, in a range of 50.7 to 55.2 g kg^{-1} DM, which did not occur with the treatments formed by KSB+50% and KSB+0% (Figure 11A). K concentration in the plant tissue was defined by the level of fertilization; K concentration increased as fertilization increased, with concentrations of 23.58, 42.22 and 23.58 g Kg^{-1} DM for 0, 50 and 100 % fertilization, respectively. The statistical differences in terms of the effect of the treatments on the K content and concentration in the plant tissue were defined by plant growth promoted by bacteria (Figure 11B).

Effect on dry matter production. Significant differences (P=0.0297) were found between the treatments of KSB+%K in terms of production of plant tissue dry matter. However, no differences were found between the treatments inoculated with KSB and the controls (Figure 10D). The KSB+100%K treatments had the lowest plant dry matter production compared to the KSB+50%K and KSB+0%K treatments. This could be due to the K solubilization by the KSB, which resulted in a K increase in the soil but with a negative effect on plant growth; this did not occur in the control. Therefore, these findings indicate that KSB should be inoculated using a fertilization rate lower than 100 %.

No statistical differences were found between the treatments (KSB+%K) in terms of root and total dry matter production. The level of fertilization (% K) was the factor that most significantly affected the production of root and total dry matter. The amount of total dry matter in the controls increased slightly as fertilization rate increased. However, dry matter content in the treatments inoculated with KSB increased slightly from 0 to 50 % K fertilization but decreased with 100 % K fertilization. The highest production of root dry matter was at 50% K fertilization (1.15 g), which corresponds to 34.8 % and 14.8 % more than the amount obtained under 0 % K and 100 % K fertilization (0.98 g), respectively, including the controls. Therefore, these results indicate that the differences in total dry matter between fertilization levels were given by root dry matter (Figure 8B). This may be influenced by the production of the ACC hormone by Tmt 32, LsC-58, Ltj-62, and by the production of AIA by LsC-21 strain, which helps promote root growth (Sepúlveda-Caamaño et al., 2018; Cedeño-Garcia et al., 2018).

Effect on K bioavailability and growth promotion. K uptake by plants is proportional to the content of readily available K in the soil, even at concentrations much higher than those required for maximum yield. The KSB+100%K treatments increased K concentrations in tomato plants above the sufficiency range (Figure 11A), so that seedling growth was affected to the extent of producing 23 % less dry matter than plants fertilized with 50 and 100 % K (Controls). Growth was not affected in the Control+100%K (Figure 11B) because the levels of available K in the soil were lower than those in the soils inoculated with KSB. This may indicate that KSB increased the available K content in the soil to the extent of exceeding the plant's requirement. There are no reports on K toxicity in plants. However, an increase in K concentration reduces the uptake of Ca, Mg and B, which indicates that there is an antagonistic effect between these elements due to the physiological properties of these ions, also affecting the synergism between K with N, Cu, Mn and Zn (Luan et al., 2009). Therefore, it can be stated that KSB promote plant growth in tomato seedlings only when 50 % of the required K is provided. Tomato seedlings require low available K content in the soil, otherwise plant growth decreases and an imbalance between soil cations occurs (Fontes et al., 2000). Levels of available K in the soil were low at 0 % and 50 % K

fertilization. Regarding 50 % K, KSB achieved the greatest growth promotion (total dry matter) and N content in the plant (Figure 11).The strains of the genus *Pseudomonas* (Tmt-32; Ls-C21 and Ltj-62) helped improve K fertility in a K-deficient soil and kept K content in tomato seedlings within the sufficiency range. *Pseudomonas* are bacteria that can adapt to several environmental conditions. In fact, they can live in any habitat with a temperature range of 4 to 42 °C, and a pH between 4 and 8; and are among the most efficient rhizosphere colonizers. (Lugtenberg and Kamilova, 2009).



Fuente: Elaboración propia.

Figure 11. Effect of the interaction between K solubilizing bacteria (KSB) and potassium fertilization levels (% K), in the relationship between: (A) concentration of available K in the soil and in the plant tissue, and (B) K and dry matter in the plant tissue of tomato seedlings.

Regarding strains belonging to the species *Acinetobacter calcoaceticus*, LsC-58 and LsC-54 applied with 50 % K fertilization helped improve K fertility levels in the soil. *A. calcoaceticus* has been reported as a growth promoter for pearl millet and cucumber (Rokhbakhsh-Zamin et al., 2011; Kang et al, 2014), and to have biological control properties against the pathogenic bacterium *Ralstonia solanacearum* in tomato (Xue et al 2009). The genus *Acinetobacter* enhances Fe, Zn, Mg, Ca, K and P uptake in

plants. Furthermore, species of this genus have been also described as N_2 fixing bacteria in sugarcane (Taulé et al., 2012).

The use of K fertilizers has increased worldwide, while the fertilizer use efficiency has decreased (Dhillon et al, 2019). KSB cannot only increase K availability in the rhizosphere, but also enhance water holding capacity of soils, and improve the structural stability in sandy soils, and can have positive effects on plant growth by suppressing pathogens (Etesami et al., 2017).

4.4.4 Compatibility between bacteria selected as NFB, PBS and KBS

The results of the compatibility test between the strains that promoted higher plant growth and greater NPK bioavailability showed that Tmt1-107, Tmt-16, Tmt-32, Ltj-62, Hb-142, Ls-C21, Ls-C58 and Ls-C54 were compatible with each other, because they did not present zones of inhibition in the growth of colonies of each strain. In the future, these strains can be tested in consortium and contribute to increasing nutrient use efficiency and crop productivity for sustainable agricultural production. Some reports have described that the application of a bacterial complex has increased the level of available N, P and K in the soil, with conclusive results in the rotation tomato-spinach, inoculated with a mixture of two strains of *Bacillus subtilis* and two strains of *B. mucilaginosus* (Song et al., 2015); in alfalfa inoculated with a mixed complex of *Azotobacter* spp. as P and K solubilizing bacteria (Han et al., 2011); and in kiwi inoculated with a bacterial complex composed of *Bacillus amyloliquefaciens*, XD-N-3 as N_2 fixing bacteria, *B. pumilus*, XD-P-1, XD-P-1 as a solubilizer of P and *B. circulans*, XD-K-2 as a K solubilizer (Shen et al., 2016).

4.5. CONCLUSIONS

The interaction between bacteria and different levels of fertilization had several effects on NPK bioavailability and growth promotion of tomato seedlings.

Each N_2 fixing bacteria (NFB), Tmt-16 *P. gessardi*, Hb-142 *P. marginalis* and Tmt1-107 *R. aquatica*, behaved differently at each level of N fertilization. Tmt-16 in a soil without N fertilization increased N content in the plant tissue (71.2 %). Strain Hb-

142 under 50%N fertilization, increased N content in the plant tissue and dry matter production by 19.8 and 29.2 %, respectively, while Tmt1-107increased dry matter production by 24.6 %. The use of these N_2 fixing bacteria together with nitrogen fertilization lower than 50 % increased N content in the plant tissue and stimulates growth. Inoculation with Tmt-16 at 100 % N fertilization improved the concentration of available NO_3^- and N in the soil by 51.6 and 43.8 %, respectively.

The bacteria evaluated as phosphorus solubilizers (PSB), LsC-58, *A. calcoaceticus,* Tmt1-107 *R. aquatica* and Hb-142 *P. marginalis*, showed no positive effect on growth or P bioavailability to tomato plants at any level of phosphate fertilization.

Potassium solubilizing bacteria (KSB), Tmt-32 and Ls-C21, *P. koreensis*, Ltj-62, *P. brassicacearum*, LsC-58 and LsC-54, *A. calcoaceticus*, applied at 100 % level of potassium fertilization, improved K availability in the soil: Ltj-62+100%K, LsC-54+50%K and LsC-21+0%K increased exchangeable and available K from 14 to 60 % in the soil. The treatments did not increase K content in the plant tissue or promote growth.

The various effects of the interaction between N_2 fixing, P solubilizing and K solubilizing bacteria with different fertilization levels offer a wide range of possibilities for the use of these rhizobacteria in soils with different fertility levels, also allowing for sustainable tomato production.

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V. CONCLUSIÓN GENERAL

Se seleccionaron *in vitro*, cuatro rizobacterias por su capacidad para fijar N_2, tres por su capacidad para solubilizar P y seis por su capacidad para solubilizar K de biotita y feldespato-K, aisladas de los cultivos de tomate, lenteja, chile, haba y lechuga cultivados en suelos Andisoles y Ultisoles. Identificadas como *Pseudomonas gessardi, P. koreensis, P. brassicacearum*, *P. Marginalis*, *Acinetobacter calcoaceticus y Rahnella aquatica*.

De las bacterias seleccionadas *in vitro*, se seleccionaron cuatro bacterias debido a su capacidad para mantener los niveles de N y K disponibles para las plantas, en un suelo Ultisol con un bajo contenido de N y K; Tmt-16, *P. gessardi*; Ls-C21, *P. koreensis*; Ltj- 62 *P. brassicacearum* y LsC-58, *A. calcoaceticus*. La rizobacteria Tmt-16 aumentó el crecimiento de las raíces, parámetro de calidad en la producción de plántulas de tomate. Las cepas solubilizadoras de P no mostraron un efecto positivo sobre el crecimiento de la planta de tomate o el aumento de P disponible en el suelo. Con base en la huella genética, ninguna de las rizobacterias tuvo la capacidad de establecerse como endófitas.

La interacción entre bacterias y distintos niveles de fertilización generó diversos efectos en la biodisponibilidad de NPK y la promoción de crecimiento de las plántulas de tomate. Cada bacteria fijadora de N_2; Tmt-16 *P. gessardi*, Hb-142 *P. marginalis* y Tmt1-107 *R. aquatilis,* respondió de manera distinta en cada nivel de fertilización nitrogenada. La aplicación de estas bacterias fijadoras de N_2 en conjunto con una fertilización nitrogenada menor al 50 %, mejora el contenido N en el tejido vegetal y estimula la promoción de crecimiento. La inoculación de Tmt-16 en conjunto con el 100 % N, mejoró la concentración de NO_3^- y N-disponible en el suelo. Las bacterias evaluadas como solubilizadoras de P; LsC-58, *A. calcoaceticus*, Tmt1-107 *R. aquatica*. y Hb-142 *P. marginalis*, no mostraron un efecto positivo en el crecimiento, ni en la biodisponibilidad de P para las plantas de tomate, en ninguno de los niveles de fertilización fosfatada. Las bacterias solubilizadoras de K; Tmt-32 y Ls-C21*, P. koreensis,* Ltj-62, *P. brassicacearum,* LsC-58 y LsC-54, *A. calcoaceticus* aplicadas en conjunto con un nivel de fertilización potásica al 100 %, mejoraron la disponibilidad de

K. En la planta, los tratamientos no indujeron mayor contenido de K en el tejido vegetal y no promovieron el crecimiento. Los diversos efectos de la interacción entre las bacterias fijadoras de N_2, solubilizadoras de P y solubilizadoras de K con los distintos niveles de fertilización, brinda una amplia posibilidad del uso de estas rizobacterias en suelos con distintos niveles de fertilidad. Permitiendo generar una alternativa sostenible para la producción del cultivo de tomate.

Las rizobacterias fueron compatibles entre sí por lo que en un futuro pueden evaluarse en consorcio y contribuir a aumentar la eficiencia en el uso de nutrientes y la productividad en diversos cultivos para una producción agrícola sostenible.