In vitro Detection and Optimization of Salicylic Acid from the Rhizobacterial Strains *Pseudomonas aeruginosa* UPMP3 and *Burkholderia cepacia* UPMB3 for Plant Defense

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Abstract

Salicylic acid (SA) produced by different plant growth-promoting rhizobacteria (PGPR) is a key phytohormone that regulates plant growth and defenses against pathogens. *Pseudomonas aeruginosa* UPMP3 and *Burkholderia cepacia* UPMB3 are the most important types of plant growth-promoting rhizobacteria isolated from oil palm rhizosphere. The aim of this study was to detect and optimize SA production by the two PGPR *in vitro*. Production of SA was extracted, purified, detected, confirmed and optimized from these two rhizobacterial strains through Thin Layer Chromatography analyses (TLC). Different parameters i.e. casamino acid, pH, temperature, static and shaken condition were considered to optimize the SA production. Salicylic acid production by the two strains was confirmed by TLC analyses, in which the R_f (Retention factor) value was 0.74 respectively that were matched with the authentic SA. Both of these Rhizobacterial strains produced SA, with a maximum yield of 16.29 and 11.13 µg/ml in casamino acids at a concentration of 0.50%, 13.13 and 10.11 µg/ml under pH 7.0, 13.14 and 10.34 µg/ml under 30°C temperature, 12.95 and 9.95 µg/ml at 150 rpm in shaking condition for 3 days incubation period respectively. Therefore, the present study indicates that the rhizobacterial strains *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 have merits to be beneficial bacteria for the plant protection inducing defense mechanism.

সারসংক্ষেপ

উদ্ভিদের বৃদ্ধি উদ্দীপক বিভিন্ন রাইজোব্যাকটেরিয়া (Plant Growth Promoting Rhizobacteria) কর্তৃক উৎপাদিত স্যালিসাইলিক এসিড (SA) একটি প্রধান ফাইটোহরমোন, যা উদ্ভিদের বৃদ্ধি এবং রোগ সৃষ্টিকারী জীবাণু বা পোকামাকড়ের আক্রমণ থেকে রক্ষার উদ্দীপক হিসেবে কাজ করে। *Pseudomonas aeruginosa* UPMP3 and *Burkholderia cepacia* UPMB3 দুইটি গুরুত্বপূর্ণ রাইজোব্যাকটেরিয়াল স্ট্রেন যা অয়েলপাম উদ্ভিদের রাইজোস্ফেয়ার থেকে শনাক্ত করা হয়েছিল। এই পরীক্ষার লক্ষ্য ছিল উল্লিখিত দুইটি রাইজোব্যাকটেরিয়া থেকে ইনভিট্রো অবস্থায় স্যালিসাইলিক এসিড শনাক্ত ও উৎপাদন নিশ্চিত করা। স্যালিসাইলিক এসিড নিষ্কাশন, বিশুদ্ধকরণ, শনাক্তকরণ এবং নিশ্চিতকরণ Thin Layer Chromatographic বিশ্লেষণের মাধ্যমে করা হয়েছিল। স্যালিসাইলিক এসিড-এর সর্বোচ্চ উৎপাদনে বিভিন্ন প্যারামিটার যেমন- কাসামিনো এসিড, পিএইচ, তাপমাত্রা, স্থির ও ঝাঁকানো অবস্থা বিবেচনা করা হয়েছিল। এই দুইটি রাইজোব্যাকটেরিয়াল স্ট্রেন হতে স্যালিসাইলিক এসিড-এর শনাক্তকরণ নিশ্চিতকরণে Thin Layer Chromatography

কৌশল দ্বারা উভয় ব্যাকটেরিয়ার R_f -এর মান ০.৭৪ পাওয়া গিয়েছিল যা প্রকৃত বা আসল SA-এর সমকক্ষ। উভয় ব্যাকটেরিয়ার ক্ষেত্রে ৩ দিনের ইনকিউবিশন সময়ে ০.৫০% কাসামিনো এসিডের ঘনত্বে SA এর সর্বোচ্চ উৎপাদন হয়েছিল যথাক্রমে– ১৬.২৯ ও ১১.১৩ মাইক্রোগ্রাম/মিলি.; ৭.০ পিএইচ এ ১৩.১৩ ও ১০.১১ মাইক্রোগ্রাম/মিলি.; ৩০° সেন্টিগ্রেড তাপমাত্রায় ১৩.১৪ ও ১০.৩৪ মাইক্রোগ্রাম/মিলি. এবং ঝাঁকানো অবস্থায় ১৫০ আরপিএম এ ১২.৯৫ ও ৯.৯৫ মাইক্রোগ্রাম/মিলি.। সুতরাং, এই পরীক্ষার ফলাফল ইঙ্গিত করে যে, *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 দুটি রাইজোব্যাকটেরিয়াল স্ট্রেন-এর মধ্যে উপকারী বা কল্যাণকর ব্যাকটেরিয়ার গুণাগুণ আছে যা স্বপ্রতিরোধি পদ্ধতির মাধ্যমে উদ্ভিদ রক্ষায় কাজ করে।

Key words: *Burkholderia cepacia* UPMB3, ***In vitro***, Plant defense, *Pseudomonas aeruginosa* UPMP3, Salicylic acid, Thin layer chromatography.

Introduction

Plant growth promoting rhizobacteria are the dominant driving forces in recycling the soil nutrients and consequently, they are crucial for soil fertility. Currently, the biological approaches for improving crop production are gaining additional interest among agronomists and environmentalists following integrated plant nutrient management system. In this context, there is an on-going rigorous research worldwide with greater impetus to explore a wide range of rhizobacteria possessing novel traits like biological control of phytopathogens and insects (Hynes *et al.* 2008) along with the normal plant growth promoting properties such as, phytohormone (Ahemad and Khan 2012c), siderophores (Tian *et al.* 2009), antibiotics (Bhattacharyya and Jha 2012), hydrogen cyanaide (HCN) and ammonia production, nitrogenase activity (Khan 2005; Glick 2012) phosphate solubilization, heavy metal detoxifying potentials (Ahemad and Khan 2012c; Ma *et al.* 2011), and pesticide degradation/ tolerance (Ahemad and Khan 2012a; b). Salicylic acid is produced in significant amounts by certain plant growth promoting rhizosphere bacteria, and some of these rhizobacteria have the ability to induce systemic resistance against diseases in plants. The application of SA to plants has long been known to lead to protection against plant pathogens through the elicitation of systemic acquired resistance (Bakker *et al.* 2014). Different *Pseudomonas* and *Burkholderia* species have emerged and potentially promising group of PGPR. Salicylic acid (SA) is an essential signal elicitor for the induction of Induced systemic resistance (ISR) and the orchestration of the events that occur during the hypersensitive response (HR) (Carl *et al.* 2005). Indhiragandhi *et al.* (2008) reported that *Acinetobacter* sp., *Pseudomonas* sp. and *Serratia* sp. shows production of salicylic acid, which is important component in the induction of defense in plants. Earlier, some previous studies reported the influence and role of salicylic acid for protection of plants under various biotic and abiotic stresses including salinity (Horvâth *et al.* 2007; Dempsey and Klessig 2017) and in growth and development (Rivas-San Vicente and Plasencia 2011).

The presence of SA in plants is well documented and for some plant species levels in excess of 1 µg per gram fresh weight have been reported (Raskin 1992). SA is required as a signaling molecule in systemic acquired resistance (SAR) that develops in plants after attack by pathogens that cause necrosis

(Durrant and Dong 2004). Systemic Acquired Resistance (SAR) is effective against a broad range of pathogens and the protection can be long-lasting. Manipulation of this induced defense mechanism thus has potential for plant protection. Exogenous application of SA or SA mimics can indeed protect plants against a range of pathogens (Oostendorp *et al.* 2001). Whereas animals and plants both respond to application of SA, effects on fungi have hardly been studied. Production of SA by bacteria has been reported frequently, in many cases related to the production of siderophores under iron limited conditions. Next to playing a role in iron acquisition the effects of SA on plants suggest that the production of this metabolite by bacteria can have a significant impact on plant-microbe interactions in the rhizosphere. Later on, many works had been done in order to substantiate the role of SA during plant-pathogen interactions and to substantiate the fact SA plays an important role in SAR induction in tobacco and cucumber plants (Malamy *et al.* 1990; Métraux *et al.* 1990). Further; many researchers established the prime role of SA in induced systemic resistance (ISR) induction (Yalpani *et al.* 1991; Audenaert *et al.* 2002). SA plays its key role in inducing both local and systemic induced resistance initiated after an immediate plant pathogenic attack (Saikia *et al.* 2003). De-meyer and Hofte (1997) showed *P. aeruginosa* can induce systemic resistance through SA production. They assumed that PGPR can trigger on activation of phenyl alamine ammonia-layase (PAL) activity leading to increased SA biosynthesis and suggested that SA may be indirectly involved in introducing ISR induced by PGPR with special reference to cucumber root disease. So, they considered SA as an important translocated signal that gets accumulated in PGPR treated host plant roots and greatly involved in inducing ISR. Siddiqui and Shaukat (2003) showed *P. aeruginosa* enhanced defense mechanism by inducing systemic resistance in tomato plants through production of SA. Thus in this study it will be worthwhile, to review the efficacy of *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 as the producer of salicylic acid in optimized condition.

Materials and Methods

Sources of the bacterial strains

Two plant growth promoting rhizobacteria *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 were collected from Plant Protection Department, Universiti Putra Malaysia. These bacterial strains were isolated from oil palm rhizosphere. Both strains have been characterized, sequenced and deposited with NCBI Gen Bank (Accession no. GQ183951 - *P. aeruginosa* strain UPMP3 and GQ183952 - *B. cepacia* strain UPMB3). The bacterial strains were also identified based on Biolog® identification system (Zaiton *et al.* 2006; Azadeh *et al.* 2010). In the current study, these bacterial strains were prepared from stock cultures stored at 4°C and subsequently sub-cultured on nutrient agar when required.

Reconfirmation of the bacterial strains using Biolog Reader

P. aeruginosa UPMP3 and *B. cepacia* UPMB3 were grown on nutrient agar (NA) medium for routine use, and maintained in Nutrient Broth (NB) medium with 15% glycerol at - 80°C for long-term storage. The bacterial isolates were identified and reconfirmed with the Biolog® identification system (version 4.2). The procedure for identification utilized 96 wells of microplate containing 95 different dried carbon

source plus control. Single colony of fresh bacteria from 24 hours old culture growing on NA was inoculated on Biolog Universal Growth (BUG) medium. The bacterial inoculation fluid were tested for turbidity and then inoculated in a GN III Biolog 96-well microliter plate with 100µL per well. The microliter plates were incubated at 30-33°C for 24 hours, and the resulting pattern of coloured wells analysed using the Microstation™ system and Biolog MicroLog™ software to give the bacterial identification.

In vitro detection of salicylic acid

Preparation of culture supernatant for SA

The rhizobacterial strains *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 were cultured on NA medium. After 24 hours incubation, the bacteria were grown again in casamino acids broth medium. The pH of the medium was adjusted to 7.0. The experiment was carried out in 250 ml Erlenmeyer flasks containing 50 ml of medium inoculated with 24 hours pre-cultured bacteria. Inoculated flasks were incubated at 34 ± 2°C on an incubator shaker at 200 rpm for 5 days in the dark condition. Thereafter, the bacterial cells were harvested by centrifugation at 8,000 rpm for 10 minutes at 4 °C and the supernatant was used for the screening and extraction of SA.

Screening of SA production

To screen SA production, the supernatant was acidified with 1 N HCl at pH 2.0. Then the supernatant was extracted with chloroform (2 × 2 ml). One ml of extract was added with 2 ml of 2M $FeCl_3$ and 1 ml of distilled water. The salicylic acid reacted with 2 M $FeCl_3$ to form a purple iron SA complex (purple iron colour indicator) with a maximum absorbance at 527 nm. The production of purple iron colour indicated the ability to produce SA of the both bacterial strains. Colour change was recorded in the spectrophotometer.

Extraction and purification of SA

Salicylic acid was extracted and purified following the method described by Shanmugam and Narayanasamy (2009). For extraction and purification of SA production, 50 ml of cell free supernatant was reduced to 20 ml by evaporation under vacuum and acidified at pH 2.0 with 1N HCl. The supernatant was extracted twice with double volume of chloroform. Extracted chloroform fraction was evaporated to dry in a rotatory evaporator at 40°C and dissolved in 1 ml of methanol. After filtering through 0.45 µm membrane filter, the extract was kept at -20°C for confirmation by TLC.

Confirmation of SA production by TLC analysis

Twenty µl of the extracted and purified SA were spotted on pre coated silica gel plates. Then the plates were developed in a solvent system consisting of chloroform: acetic acid: ethanol at the ratio of 95: 2.5: 2.5 (v/v). The plates were viewed under UV light (254 nm and 365 nm) immediately after removal from the developing chamber. The SA was detected by observing a UV reflected band with an R_f value corresponding to that of standard SA.

Optimization and Quantification of SA Production

The efficiency of *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 to produce SA was tested in casamino acids medium with different concentrations of casamino acids (0.10-0.70%) at different pH (3-9), temperature (20-45°C) and in static and shaken conditions (50-250 rpm). The broth cultures were incubated for 5 days and cells were separated by centrifugation at 8000 rpm for 10 minutes. The supernatant was collected and SA was measured spectrophotometrically at 530 nm. All the

harvesting procedures were carried out in dim light with samples maintained in covered ice baths.

Effect of casamino acid concentration on SA production

To check the effect of casamino acid on SA production, casamino acid was prepared with different concentration (0.10-0.70%) and inoculated with the selected strains. 1% inoculum of optical density $(O.D)_{600}$ 1.0 was incubated for 1-5 days respectively. After incubation the broth was centrifuged at 8000 rpm for 10 minutes. The supernatant was collected and measured SA for quantification.

Effect of pH on SA production

To optimize different pH level on SA production by the strains UPMP3 and UPMB3, selected concentration of casamino acid medium was adjusted to pH as 3, 4, 5, 6, 7, 8 and 9. Medium was inoculated with 1% inoculum and incubated for 1-5 days respectively. After incubation, the broth was centrifuged at 8000 rpm for 10 minutes. The supernatant was collected and measured SA for quantification.

Effect of culture conditions on SA production

To optimize different culture conditions on SA production, bacterial strains were grown in selected concentration of casamino acid medium at a range of 1-5 days in static and shaken (50, 100, 150, 200, and 250 rpm) conditions at 28±2°C on incubator shaker respectively. After incubation the broth was centrifuged at 8000 rpm for 10 minutes and collected the supernatant. Then SA was measured for quantification.

Effect of temperature on SA production

To optimize the temperature on SA production by the bacterial strains were grown in the selected concentration of casamino acid medium at a range of 1-5 days in different temperature (25, 30, 35, 40 and 45°C) on incubator shaker. After incubation the broth was centrifuged at 8000 rpm for 10 minutes. The supernatant was collected and SA was measured for quantification.

Statistical analysis

All experiments were performed as Completely Randomized Design (CRD). Data were analysed using statistical analysis system (SAS v9.3) and means were statistically compared using LSD test. The significance level was set up at p <0.05. Three replications were considered for each treatment and repeated twice.

Results

Confirmation of bacterial strains using Biolog reader system

P. aeruginosa UPMP3 and *B. cepacia* UPMB3 were confirmed with the Biolog® identification system and categorized into *P. aeruginosa* and *B. cepacia* taxonomic groups. The bacterial strains were identified as shown in Table 1 and Fig. 1.

Table 1. Identification and reconfirmation of *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 from Biolog® identification system.

Rhizobacterial strain	Probability	Similarity	Distance	Type
P. aeruginosa UPMP3	100	0.57	6.68	GN-NENT
B. cepacia UPMB3	100	0.84	2.83	GN-NENT

Based on Biolog® identification system the bacteria UPMP3 was identified as *P. aeruginosa* and UPMB3 as *B. cepacia* with similarity reading of 0.57 and 0.84 respectively. Probability values were both 100.

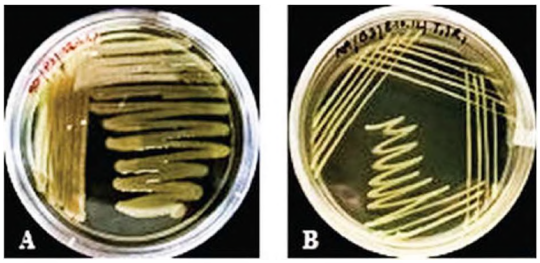


Figure 1. Bacterial strains on culture media. A: *P. aeruginosa* UPMP3 B: *B. cepacia* UPMB3.

Screening and confirmation of SA production by TLC

The bacterial strains were screened for SA production. *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 showed purple iron colour react with $FeCl_3$ which indicated their ability to produce SA (Fig. 2).

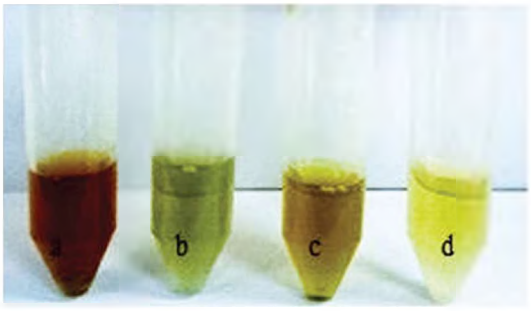


Figure 2. Screening of SA produced by P. *aeruginosa* UPMP3 and *B. cepacia* UPMB3. a: UPMP3 (purple iron colour), b: UPMP3 (control), c: UPMB3 (purple iron colour), d: UPMB3 (control).

Detection and confirmation of SA production by TLC were carried out with the appearance of blue bands matched with that of authentic SA bands on pre-coated silica gel plates under UV illumination at 365 nm. The R_f value was found 0.74 both for *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 that was similar to the R_f value (0.74) of the authentic SA (Fig. 3).

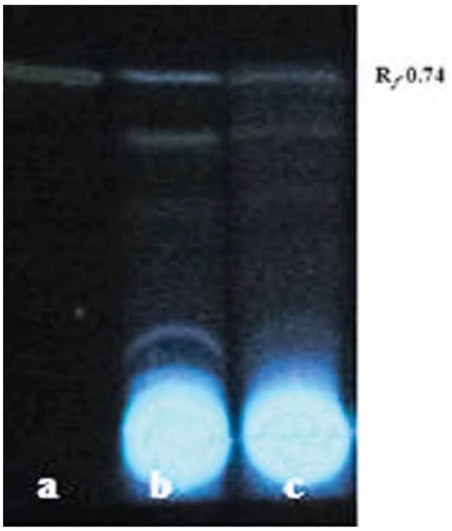


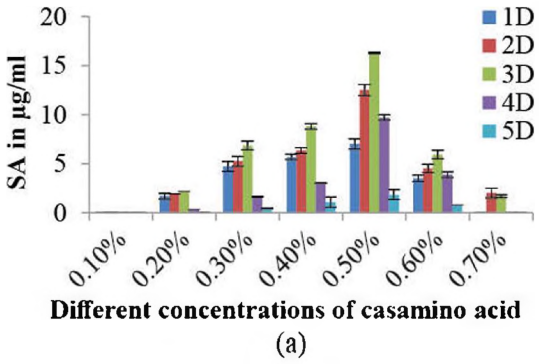
Figure 3. Detection of SA by TLC, a: Standard SA, b: UPMP3, c: UPMB3.

Optimization and Quantification of Salicylic Acid

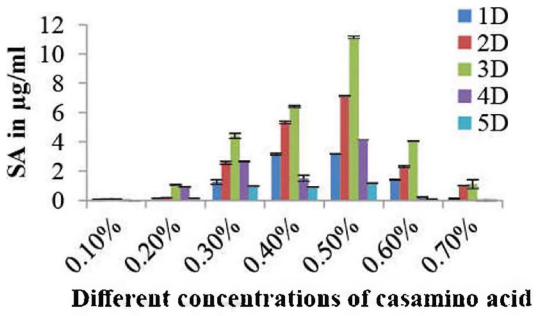
The optimal conditions for the SA production by UPMP3 and UPMB3 were standardized.

Effect of casamino acid concentration on SA production

Different concentrations of casamino acid as a substrate ranging from 0.10 - 0.70% was tested for the production of SA from UPMP3 and UPMB3. The optimum SA production was found 16.29 µg/ml from UPMP3 and 11.13 µg/ml from UPMB3 in 0.50% casamino acid concentration on the 3rd days of incubation period (Fig. 4a &4b).

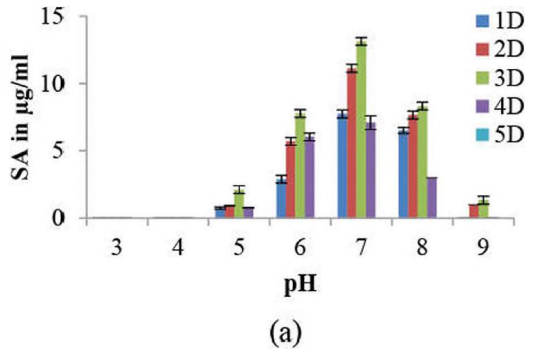


(a)

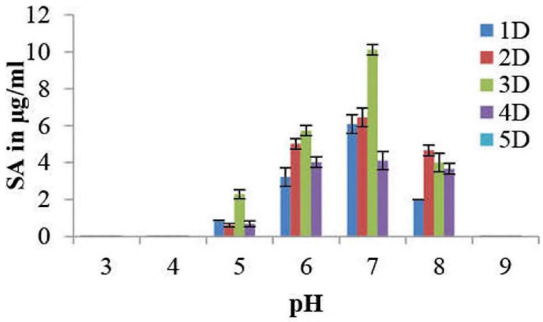


(b)

Figure 4. Production of salicylic acid by UPMP3 (a) and UPMB3 (b) at different concentration of casamino acid. Vertical bars represent standard error.



(a)



(b)

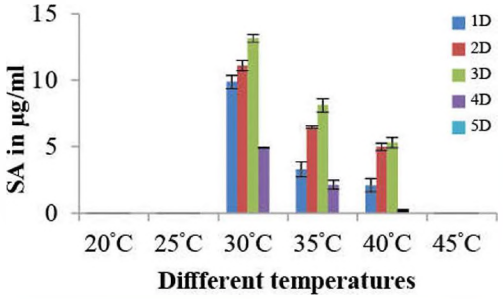
Figure 5. Production of salicylic acid by UPMP3 (a) and UPMB3 (b) at different pH. Vertical bars represent standard error.

Effect of pH on SA production

Among different pH tested, the pH 7.0 favoured the maximum SA production of 13.13 µg/ml from UPMP3 and 10.11 µg/ml from UPMB3 as against 2 µg/ml -8 µg/ml in rests of the pH on the 3rd days of incubation period (Fig. 5a & 5b).

Effect of Temperature on SA production

Among different temperature tested, P. aeruginosa UPMP3 and B. cepacia UPMB3 produced maximum SA of 13.14 µg/ml and 10.34 µg/ml respectively at 30°C on the 3rd day of incubation period. The SA production in rest of the temperature ranged between 2 µg/ml - 11 µg/ml. It has been observed that in both the lower and higher temperature (20, 25, and 45° C), there was no SA production (Fig. 6a & 6b).



(a)

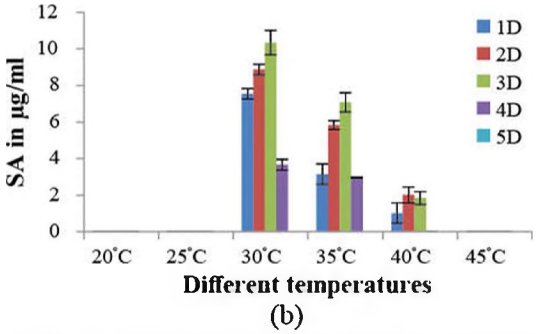


Figure 6. Production of salicylic acid by UPMP3 (a) and UPMB3 (b) at different temperatures. Vertical bars represent standard error.

Effect of culture condition on SA production

Among the static and shaken conditions, culturing *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 at shaken condition with 150 rpm was found to be optimum for the production of SA 12.95 µg/ml and 9.95 µg/ml successively on the 3rd days of incubation period than static condition (Fig. 7a & 7b).

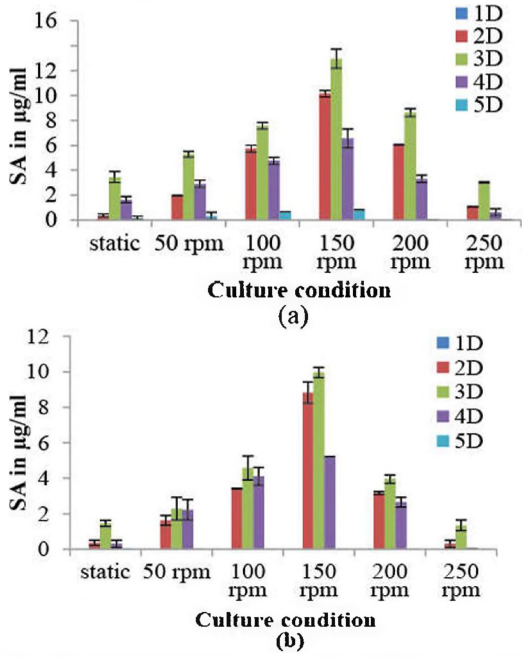


Figure 7. Production of salicylic acid by UPMP3 (a) and UPMB3 (b) at static and shaken conditions. Vertical bars represent standard error.

Discussion

Salicylic acid is known to play major roles in regulating plant defense responses against various pathogens, pests and abiotic stresses such as wounding and exposure to ozone (Balbi and Devoto 2008). It is generally involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens as well as the establishment of systemic acquired resistance (SAR). Recently, it has been shown that, methyl salicylate, which is induced upon pathogen infection, acts as a mobile inducer of SAR in tobacco (Park *et al.* 2007). SA levels increase in pathogen challenged tissues of plants and exogenous applications result in the induction of pathogenesis related (PR) genes and enhanced resistance to a broad range of pathogens. The PGPR activity and its potential capability of salicylic acid production for sustainable plant protection is well established (Parvin *et al.* 2015). SA in the culture filtrate of the *P. aeruginosa* IE-6S^{+} strain was detected in TLC as a blue spot at an R_f value of 0.91 after exposure to ammonia fumes. *In vitro* SA production by *P. aeruginosa* IE-6S^{+}, determined spectrophotometrically, revealed that the bacterial inoculant cultivated in casamino acid liquid medium synthesized SA at 3.9 ± 1.1µg/ml (Imran and Shaukat 2003). *Bacillus licheniformis* MML2501 produced SA that was confirmed by the blue bands that appeared on pre-coated silica gel viewed under UV illumination and the R_f value was 0.61 which was similar to the authentic SA (Shanmugam and Narayanasamy 2009). In order to determine the ability of the B. licheniformis MML2501 for the production of salicylic acid, experiments were conducted under *in vitro* and *in vivo* conditions. Under optimal pH, temperature, concentration of substrate and shaken conditions, *B. licheniformis* MML2501 showed maximum

production of 18 µg/ml of SA, which is important component in the induction of plant mediated defense enzymes (Shanmugam and Narayanasamy 2009).

The antagonistic potential of the isolated purple non sulfur bacterium (PNSB) *Rubrivax gelatinosus* RASN4 strain was tested in terms of their potentiality of SA production (*in vitro*), which showed a strong positive indication through screening prior to their quantification. During estimation, the amount of *in vitro* SA production by PNSB strain RASN4 was determined up to 27.3 mg/l as maximum highest level (Gupta and Sinha 2020). In TLC, appearance of blue bands both in case of samples and authentic SA standard control, showing same fluorescence postulated strongly the capability of *in vitro* bacterial SA production potentials of isolated rice rhizospheric PNSB RASN4 *Rubrivax gelatinosus* strain (Gupta and Sinha 2020). Production of SA is also influenced by temperature. Ran *et al.* (2005) reported that the relatively high production of SA *in vitro* by *P. fluorescens* strain WCS374 is even enhanced at supra-optimal temperatures (i.e. 31–33°C). This increase was also observed for *P. fluorescens* CHA0r, but not for *P. fluorescens* WCS417r or *P. aeruginosa* 7NSK2. Similarly salicylic acid has been found synthesized in the culture supernatants of *Pseudomonas aeruginosa* and *Pseudomonas cepacia* (Viska *et al.* 1993). Similar effect of temperature (30 °C) on SA production was observed in this study. Indiragandhi *et al.* (2008) also reported that *Serratia* sp. PSGB13, *Acinetobacter* sp. PRGB16 and *Pseudomonas* sp. PRGB06 produces extra cellular salicylic acid with the concentration of 10.0 ± 0.7, 7.2 ± 0.6 and 6.8 ± 0.4 g/ml respectively. De Meyer and Hofte (1997) stated that some plant growth promoting bacteria (PGPB) do trigger a salicylic acid dependent signaling pathway by producing small amount of salicylic acid in rhizosphere. Apart from the production of secondary metabolites, induced systemic resistance in plants by rhizobacteria may also be attributed to the diseases suppression. ISR mediation through salicylic acid is already well established (Van loon *et al.* 1998). Enhancement of induced disease resistance by salicylic acid dependent pathways against bacterial pathogen was carried out in *Arabidopsis thaliana* (Van Wees *et al.* 2000). *Pseudomonas* is the best studied genus for SA production and SA-producing species include *Pseudomonas aeruginosa*, *P. aureofaciens*, *P. corrugata* and *P. fluorescens*. *P. fluorescens* holds the largest number of SA-producers studied, including strains WCS374 and WCS417, CHA0, Pf4–92, Pf12–94, Pf151–94 and Pf179–94 or PICF3, PICF4 and PICF7 (Saikia *et al.* 2003; Mercado-Blanco *et al.* 2004). The latter strains were reported to produce only minor amounts of SA when grown in standard succinate medium (SSM) (Mercado-Blanco *et al.* 2004). Among them, the olive (*Olea europaea* L.) root endophytic strain PICF7 has been shown to be an efficient biological control agent (BCA) against Verticillium wilt of olive (*Verticillium dahliae*) and able to trigger a broad range of defense responses in olive root tissues (Schilirò *et al.* 2012). *Pseudomonas fluorescens* strains WCS374 and WCS417, isolated from potato (*Solanum tuberosum* L.) and wheat (*Triticum aestivum* L.) rhizospheres respectively have been investigated for plant growth promotion and biocontrol activities in several plant species and against diverse pathogens (Bakker *et al.* 2007a; De Vleesschauwer and Höfte 2009). *In vitro* production of SA by strains WCS374 and WCS417 was measured in SSM with low iron availability. Strain WCS374 can

be considered as a SA 'super-producer' (up to 55 µg per ml) *in vitro*. This amount is approximately 10 times higher than that detected for WCS417 as well as for other SA producers under similar culturing conditions (Leeman *et al.* 1996). *P. fluorescens* CHA0, a well-studied PGPR strain, was originally isolated from roots of tobacco (*Nicotiana tabacum* L.) plants grown in soil naturally suppressive to black root rot, a disease caused by *Thielaviopsis basicola* (Stutz *et al.* 1986). For this strain *in vitro* SA production was detected under low iron conditions and effects of carbon sources and minerals on production have been investigated (Duffy and Défago 1999). At elevated temperature, SA production by CHA0 is enhanced (Ran *et al.* 2005a). Besides SA-producing rhizosphere pseudomonads, other bacterial genera have been demonstrated to produce SA. *Serratia marcescens* strain 90–166 was also characterized as an SA-producing rhizobacterium. SA biosynthesis by strain 90–166 is affected by the culture medium with the highest production in Kings medium B that has low iron availability (Zhang *et al.* 2002). Whereas measuring bacterial SA production in vitro is rather straightforward, detection of bacterial SA in the rhizosphere is challenging. On cucumber roots colonized by *P. aureofaciens* 63–28 or *P. corrugata* 13, Chen *et al.* (1999) measured elevated levels of SA as compared to control roots. However, the elevated levels were magnitudes higher than those produced by the bacteria *in vitro*, and the authors concluded that the bacteria stimulated the plant itself to accumulate SA.

Many PGPR have the ability to produce SA in an iron availability dependent way and SA is detected on plant roots (Hayat *et al.* 2013), although likely originating from plant root tissues upon interaction with rhizobacteria. Given the fact that several PGPR can elicit induced systemic resistance (ISR) in plants (Kloepper *et al.* 2004) and that application of SA to plants leads to induced resistance against a range of pathogens (White 1979; An and Mou 2011). Like all other PGPR bacterial strains *Pseudomonas aeruginosa* (Siddiqui and Shaukat 2003), *Bacillus licheniformis* (Shanmugam and Narayanasamy 2009), *B. cereus, B. mycoides, B. pumilus, B. sphaericus* and *B. subtilis* (Abdel-Monaim 2017; Saikia *et al.* 2003) *Pseudomonous fluorescens*, S*erratia marcesceus (*Zhang *et al.* 2002), many PNSB rhizobacterial strains can show the capability of SA production. Parvin *et al.* (2015) confirmed the potentiality of PGPR bacterial strains *Pseudomonas aeruginosa* UPMP3 *Burkholderia cepacia* UPMB3 for synthesizing salicylic acid (SA) *in vitro* and also proved their role in oil palm seedling growth and development. In present study, prime focus was emphasized on plant growth promoting rhizobacterial strains *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 to assess its potentiality for *in vitro* SA production as it show promising plant growth promoting rhizobacterial activity in order to bioformulate a potent microbial biofertilizer bioinoculant together with some phytopathoremidiatory traits. This study also indicated that the rhizobacterial strain UPMP3 and UPMB3 as a high salicylic acid producing organism has the merits to be explored for its ISR mediated defense induction in plants and can also be used in the xenobiotic environment.

Conclusion

In recent years, salicylic acids (SA) of microbial origin are being used as an extensive strategy in order to protect the plants from plant pathogens to mitigate plant disease for its control. Salicylic acid, exogenously produced

by the bacterial microorganisms, plays a pivotal role in plant growth and development together with the enhancement of their crop productivity through their phytopatho-remediatory effects inducing system resistance against a wide range of fungal and bacterial phytopathogens. Such capability of exogenous Salicylic acid production by the strain UPMP3 of *P. aeruginosa* and UPMB3 of *B. cepacia* might have been exploited for bio formulating an efficient microbial biofertilizer with additional potentiality of phytopathore-mediation in addition to their other PGPR activity traits. This will provide an additional synergistic advantage for bio formulating a potential microbial bioinoculant for promising agricultural crop productivity in Indian subcontinent.

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