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BIOCHEMICAL CHARACTERIZATION OF A LANTHANUM-ELICITED INSECTICIDAL COMPOUND IN ACTINOBACTERIA

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ABSTRACT

This study sought to determine the effect of Lanthanum elicitation on the insecticidal activity of *Streptomyces angustmyceticus* strain CGS B11 on the Asian corn borer (ACB), *Ostrinia furnacalis*. Lanthanum belongs to one of the rare earth elements and is found effective in eliciting secondary metabolite production in Actinobacteria. CGS B11 was isolated from volcanic soil collected in Daraga, Albay Province, Philippines. Bioassay-guided fractionation of crude extracts from CGS B11 revealed two compounds that were of comparable insecticidal activity to Spinosad. Lanthanum elicitation in CGS B11 produced one unique compound with Retention factor= 0.33 as detected by thin-layer chromatography. The compound has significantly enhanced insecticidal activity with median lethal concentration (LC_{50}) = 0.41 ng/cm^2 to neonate larvae of *O. furnacalis*. The relative potency of the Lanthanum-elicited insecticidal compound is more than 20 times greaterthan Spinosad (LC_{50}= 9.25 ng/cm^2). Further analysis by Fourier Transform Infrared Spectroscopy detected a peak at 3365.93 cm^{-1}, which indicated the presence of hydroxyl group in crude extract derived from Lanthanum-treated CGS B11. Taken together, the results of this study showed that bioprospecting from volcanic soil coupled with the use of rare earth elicitation with Lanthanum proved to be a very effective approach in enhancing potency of a potentially new insecticidal compound in strain CGS B11. The new information revealed by this study opens the avenue of using rare earths as a feasible method of eliciting new mode of action insecticides from Actinobacteria for application in insect resistance management.

Key words: *Streptomyces*, activation, fermentation, Fourier Transform Infrared Spectroscopy, bioassay

INTRODUCTION

Actinobacteria are a group of Gram-positive bacteria found in terrestrial and aquatic ecosystems (Anandan et al. 2016). They play important ecological roles such as nutrient recycling, degradation of complex polymers and production of bioactive molecules (van der Meij et al. 2017, Bhatti et al. 2017). Most of the reported bioactivities of Actinobacteria focusedon natural products with pharmaceutical and other industrial applications (Thatoi et al. 2013). However, Actinobacteria are also capable of producing environment-friendly insecticidal compounds (Kirst 2010; Barka et al. 2016). Avermectin from *S. avermitilis* (Lasota and Dybas 1991) and spinosyns from *Saccharopolyspora spinosa* (Kirst 2010) are classic examples. In the Philippines, Actinobacteria is a neglected resource of natural products for insect pest management. To date, only a few strains collected from various sources in the country were reported to contain insecticidal compounds (Bayot-Custodio et al. 2014; Creencia et al.

2021). Other reported local studies are focused on antimicrobial properties of Actinobacteria (Basilio et al. 2003; Parungao et al. 2007; Zulaybar et al. 2017).

Bioprospecting for culturable Actinobacteria from neglected or extreme habitats is reported to be an effective strategy for discovery of novel bioactive compounds (Goodfellow and Fiedler2010). Such approach reduces the chance of rediscovery of previously known compounds from commonly sampled environments (Hassan et al. 2019). High actinobacterial diversity was observed in volcanic caves (Riquelme et al. 2015). It is a specific habitat that has high potential for the isolation of new bioactive compounds (Selim et al. 2021).

Antimicrobial compounds were also discovered from Actinobacteria isolated from volcanicsoil (Cheah et al. 2015, Jia et al. 2016). Chemical elicitation with rare earth elements (REEs) was used mainly for elicitation and overproduction of antimicrobial compounds in Actinobacteria (Antoraz et al. 2015, Begani et al. 2018). The REEs consist of 17 elements including Lanthanum in the periodic table (Ochi et al. 2014). Lanthanum is effective in activating silent and poorly expressed secondary metabolite biosynthetic genes in Actinobacteria (Ochi and Hosaka 2013). The merit of the said approach is that rare earth elicitation does not require genetic manipulation to activate genes responsible for coding secondary metabolite production in Actinobacteria (Ochi 2016). Rare earths were not used for elicitation of natural insecticides in Actinobacteria and thus, should be a feasible approach to facilitate the discovery of new mode of action insecticides.

The Asian corn borer (ACB) is a major insect pest of corn in the Philippines. Reported yield losses from ACB infestation in traditional (i.e., non-*Bacillus thuringiensis* (Bt) corn) ranged from 20% to 80% (Mutuc et al. 2011). Farmers continue to use chemical insecticide application regardless of corn variety they are growing for fear of ACB or other insect pest damage (Afidchao et al. 2014). This practice, if not done judiciously could lead to insecticide resistance in ACB.

The present study sought to determine the effectiveness of Lanthanum elicitation on the production of insecticidal compound in *S. angustmyceticus* CGS B11 and to measure improvement in insecticidal activity of elicited insecticidal compound to ACB larvae.

MATERIALS AND METHODS

Isolation of Actinobacteria. *S. angustmyceticus* strain CGS B11, henceforth referred to as CGS B11 was isolated from volcanic soil in Albay Province in 2018. Briefly, approximately 100 g soil sample was collected from each of ten sites situated in one location in Cagsawa, Albay, Philippines (13º.09'55.29"N, 123º42'03.2"E). The distance between any two sites was at least one kilometer. Soil samples were collected using hand shovel and immediately placed inside sterile plastic bags. All soil samples were taken from the superficial horizon (0-30 cm depth) because the most intense microbial activity is present within this range of soil depth (Joux et al. 2015). Afterwards, the soil samples were transported to the laboratory for aseptic isolation of Actinobacteria. One gram of each soil sample was suspended in 9 ml of sterile distilled water. The suspension was thoroughly mixed using a vortex mixer and serially diluted up to 10^{-4}. An aliquot of 10^{-4} dilution was spread on yeast malt extract agar plates and incubated at 30ºC for 7 days. The composition of YMA is as follows (g/L): yeast extract 4; malt extract 10; dextrose 4; agar 20. YMA medium was sterilized by autoclaving for 25 min at 121ºC (Gao et al. 2009). To ascertain purity, colonies of CGS B11 appearing on the Petri plate after three days of incubation were re-streaked on fresh Petri plates containing the same culture medium. Each pure colony was picked from the Petri plate and streaked onto YMA test tube slant and afterwards stored at 4ºC. The identification of CGS B11 was reported in a previous study (Jimenez et al. 2021).

Chemical elicitation of insecticidal activity. A 10% inoculum was prepared by aseptically transferring a loopful of CGS B11 from YMA test tube slant into 100 ml of yeast malt extract broth (YMB), pH 7.0.

YMB is composed of the following ingredients (g/L): yeast extract 4; malt extract 10; dextrose 4; distilled water 1 L. Before use, YMB was sterilized as described above. The inoculum was cultivated in a shaker with 250 rpm agitation speed for 5 days under ambient temperature. Afterwards, the 5 day-old CGS B11 inoculum was aseptically transferred into 900 ml of YMB, pH 7.0 containing 200 μM Lanthanum which was cultivated for another 5 days at 30ºC in a shaker with 250 rpm agitation speed. Simultaneous setup with similar cultivation conditions was provided as negative control except that CGS B11 was cultivated in YMB without Lanthanum. After 5 days of cultivation, crude extract from fermentation broth from each of the two treatments was prepared by organic solvent (ethyl acetate) extraction.

Preparation of crude extracts. The fermentation broths from the two treatments (i.e., Lanthanum-treated and untreated CGS B11) were separately mixed with equal volume of ethyl acetate. The mixture was poured into a separatory funnel and manually shaken vigorously for one hour for complete extraction. The mixture was allowed to stabilize for 24 h for the separation of both the aqueous and organic phase. Afterwards, the ethyl acetate layer was collected and concentrated by evaporation using a rotary evaporator. The dried crude extract was resuspended in 2 ml methanol and transferred into amber glass vial for storage at -20°C. The methanol solution of crude extracts prepared from Lanthanum-treated and untreated CGS B11 were separately divide into equal amounts and subjected to thin layerchromatography (TLC) for secondary metabolite profiling and insecticide assay of ACB larvae. For the insect bioassay, the methanol solution of crude extract was subjected to drying using a rotary evaporator and resuspended into 2 ml distilled water containing 0.5% dimethyl sulfoxide (DMSO).

Thin-Layer Chromatography (TLC). The mobile phase (toluene: acetone: methanol, 7:3:1 v/v/v) was poured into the TLC chamber to a level a few centimeters above the chamber bottom. Individual aliquot of ethyl acetate crude extract solution was separately blotted on a TLC plate precoated with silica gel 60 as adsorbent (Merk Millipore). The TLC plate with a thickness of 200 μm in 20 cm x 20 cm format was then placed in the TLC chamber such that the sample spots were well above the level of the mobile phase. The chamber was then covered with a lid. Sufficient time was allowed for the separation of compounds contained in each spotted sample. The plate was then removed and allowed to dry. Separated sample components were viewed under the UV light at wavelength 254 nm and 366 nm.

Fourier transform infrared (FTIR) spectral analysis. Ethyl acetate crude extracts were prepared as described above. The freeze-dried samples were separately analyzed with a Shimadzu IR Prestige-21 FTIR spectrophotometer with attenuated total reflectance accessory. The spectrum was plotted as percent transmittance (% T) versus intensity of infrared spectra. Analysis was conducted at the Forest Products Research and Development Institute, Los Baños, Laguna. IR regions were analyzed using standards available at http://www.spec-online.de.

Insect bioassays. Laboratory-reared neonate ACB larvae were used for the concentration-mortality bioassays. The samples used for bioassays were the two TLC-fractionated compounds from Lanthanum-treated and untreated CGS B11, respectively. The commercial insecticide, Spinosad was included in a separate concentration-mortality bioassay to serve as reference. Six serial dilutions in logarithmic scale of each sample were prepared in 0.5% DMSO. Each of the concentration-mortality bioassays was also provide with blank buffer containing only 0.5% DMSO as negative control. An aliquot (40 ul) of each dilution was applied on the air-dried surface of artificial diet contained inside a 20-ml glass drum vial. One neonate larva was carefully placed with camel hair-brush inside the glass drum vial containing the insecticide treated artificial diet. Ten larvae were used for each concentration (i.e, dilution) . Each concentration was prepared in duplicate (Park et al. 2009; Horstmann and Sonneck 2016). Mortality was recorded after24 h. PriProbit software (Sakuma 1998) was used to estimate median lethal concentration (LC_{50}) and relative potency. When applicable, data were corrected for control mortality using Abbot's formula (Abbot 1925).

RESULTS AND DISCUSSION

Source and description of Actinobacteria. The identification of insecticidal strains of Actinobacteria is very important as it will lead to the discovery and development of these new strains as additional tool for the management of an economically important insect pest such as the ACB. One promising strain, CGS B11, previously identified as *Streptomyces angustmyceticus* (Jimenez et al. 2021) was isolated from volcanic soil collected from Cagsawa, Albay Province. Previous studies have reported that the genus *Streptomyces* is the dominant taxa in Actinobacteria (Anandan et al. 2016). The morphological features of CGS B11 is shown in Figure 1. Colonies growing on solid medium appeared as opaque, irregularly shaped, alabaster colored colonies with rough surface, umbonate elevation and wavy margin. Culture conditions greatly affect colony morphology in Actinobacteria (Li et al. 2016).

Fig. 1. Colonies of *Streptomyces angustmyceticus* strain CGS B11 growing on yeast malt extract agar medium.

Fourier Transform Infrared Spectroscopy (FTIR) analysis. FTIR spectra of ethyl acetate crude extract prepared from Lanthanum-treated CGS B11 is shown in Figure 2. The spectra revealed the presence of various characteristic functional groups through the absorption bands of Lanthanum-treated crude extract which were not observed in the untreated crude extract sample.

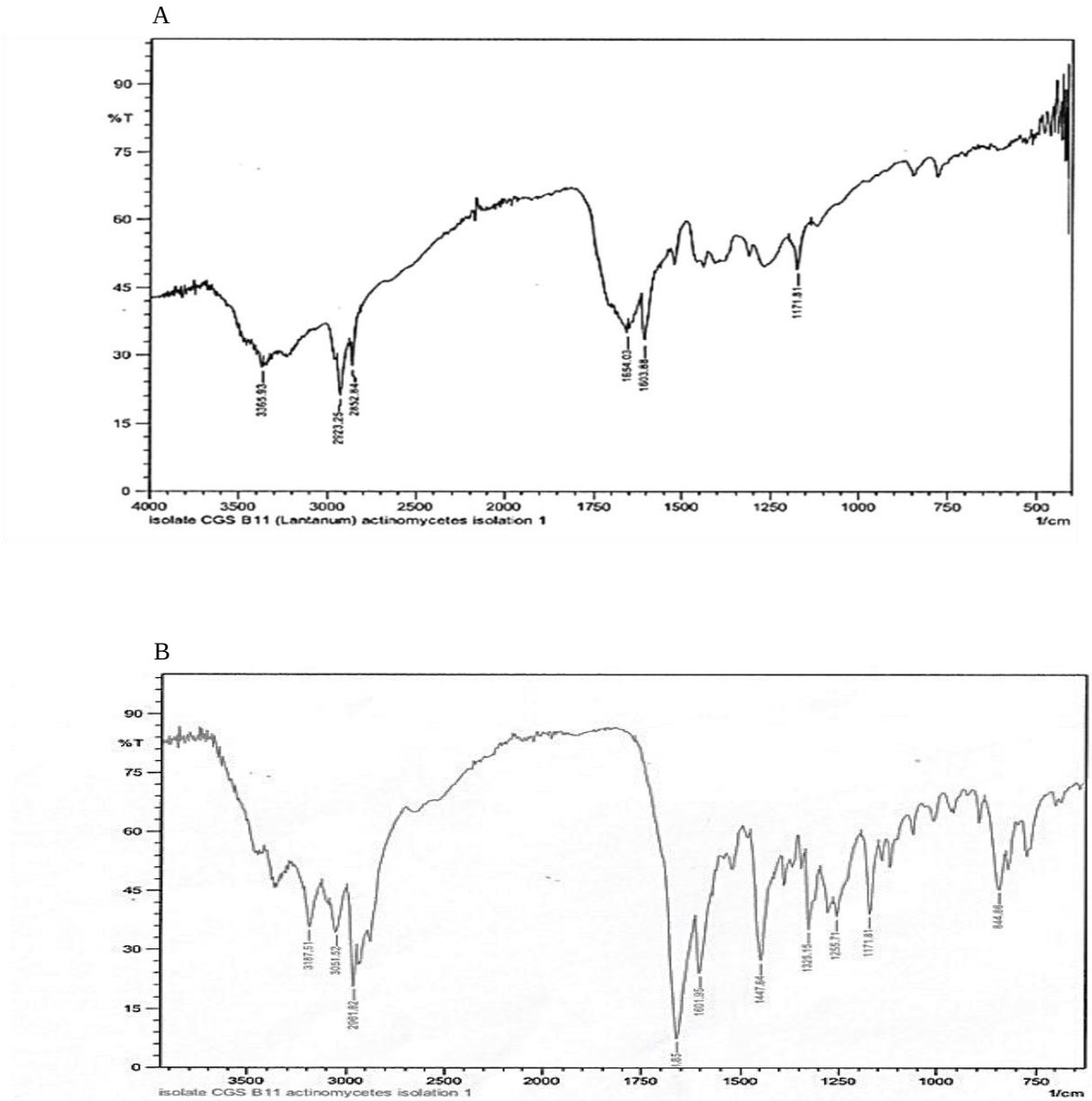

Fig. 2. Fourier transform infrared spectroscopy spectra of ethyl acetate crude extracts prepared from 5-day old fermentation broth of *Streptomyces angustmyceticus* strain CGS B11. A, Lanthanum-treated; B, untreated control.

The spectrum of Lanthanum-treated sample showed characteristic absorption bands for hydroxylgroup (H-bonded OH stretch) at 3,365.93 cm^{-1}, alkenyl group at 1,654.03 cm^{-1} (C=C stretch), methylene group (C-H asym./sym. Stretch), and primary amine at 1,603.88 cm^{-1} (Table 1).

Table 1. FTIR peaks and their respective assigned functional groups detected from dried crude extracts prepared from fermentation broh of *Streptomyces angustmyceticus* strain CGS B11.

Sample: Untreated crude extract		
Peak (cm^{-1})	Stretching and bending vibration	Assigned functional group
3187.51	Ammonium ion	
3051.52	Ammonium ion	
2961.82	C-H asym./sym. stretch	Methyl
1658.85	C-C stretch	Alkenyl
1601.95	C=C-C aromatic ring stretch	Aryl
1447.64	C-H bend	Methylene
1325.15	OH bend	Phenol or tertiary alcohol
1255.71	Aryl -O stretch	Aromatic ether
1171.81	CN stretch	Secondary amine
844.86	C-H 1,4-Distribution (para)	Aromatic ring
Sample: Lanthanum-treated crude extract		
Peak (cm^{-1})	Stretching and bending vibration	Assigned functional group
3365.93	H-bonded OH Stretch	Hydroxyl
2923.25	C-H asym./sym. Stretch	Methyl/Methylene
2852.84	C-H asym./sym. Stretch	Methyl/Methylene
1654.03	C=C stretch	Alkenyl
1603.88	NH bend	Primary amine
1171.81	CN stretch	Secondary amine

Functional groups detected only in untreated sample were as follows: aryl (C=C-C aromatic ring stretch) at 1,601.95 cm^{-1}, phenol or tertiary alcohol (OH bend) at 1,325.15 cm^{-1}, aromatic ether (-O stretch) at 1,255.71 cm^{-1}, aromatic ring (C-H 1,4-Distribution para) at 844.86 cm^{-1}, and methylene (C-H bend) at 1,447.64 cm^{-1}. FTIR analysis had been used in combination with other analytical techniques to study the antimicrobial property of Actinobacteria (Devi and Usha 2020). Similarly, the results reported here highlighted the effectiveness of FTIR as a powerful analytical method in detecting and differentiating the presence of unique functional group found only in the highly potent natural insecticide from CGS B11. Further, the results obtained from FTIR analysis support the findings of thin layer chromatography and insect bioassays of the Lanthanum-treated CGS B11 crude extracts.

Thin Layer Chromatography (TLC) Analysis. TLC profile of two ethyl acetate crude extracts of CGS B11 is shown in Figure 3. Two compounds are clearly visible in Lane 1 (control) and Lane 2 (treated) of the TLC plate chromatogram. The retention factor (Rf) of the two compounds in Lane 1 were Rf= 0.73 and Rf= 0.90, respectively. One compound with Rf= 0.33 (henceforth described as CGSB11-C1La) was detected only in Lanthanum treated crude extract (Lane 2). A second compound with Rf =0.90 (henceforth described as CGSB11-C2La) was also detected in Lanthanum-treated CGS B11. It is unclear if this compound is identical in both samples.

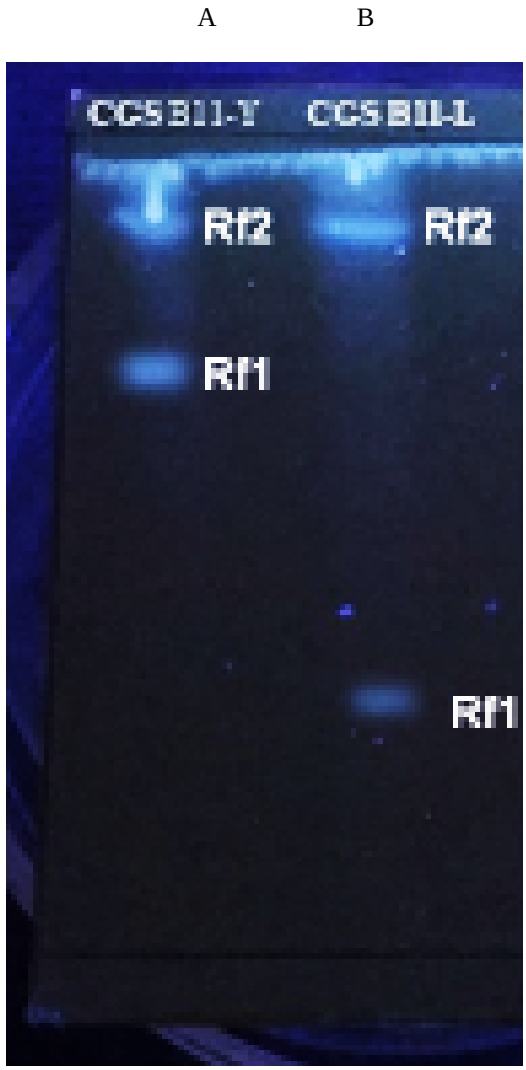

Fig. 3. Thin layer chromatography profile of Lanthanum-treated *Streptomyces angustmyceticus* strain CGS B11. Lane A: R_f1= 0.73, R_f2= 0.90; Lane B: R_f1= 0.33, R_f2= 0.90.

Insect bioassays. Two compounds detected by TLC from untreated crude extract contained insecticidal activity to neonate larvae of ACB (Table 2). The insecticidal activity of both compound 1 (LC_{50}= 1.97 ng/cm^2) and compound 2 (LC_{50}= 3.21ng/cm^2) were not significantly different from that of Spinosad (LC_{50}= 9.25 ng/cm^2). In contrast,the insecticidal activity of CGSB11-C1La (LC_{50} = 0.41 ng/cm^2) and CGSB11-C2La (LC_{50} =1.36 ng/cm^2) were significantly higher than the insecticidal activity of Spinosad (LC_{50}= 9.25 ng/cm^2).

Table 2. Probit analysis of mortality data from bioassay of purified fractions of Actinomycetes strains to Asian corn borer, *Ostrinia furnacalis*.[1]

			Model parameters		Lethal concentrations		Model fit		
Source strain	**Treatment[2]**	**n**	**intercept±SE**	**slope±SE**	**LC_{50} (95% FL) (ng/cm^2)**	**LC_{90} (95% FL) (ng/cm^2)**	**χ^2**	**df**	***P***
Streptomyces angustmyceticus CGS B11	C1-YMA	100	-0.25±0.17	0.85±0.16	1.97 (0.74-4.89)	63.61 (22.88-372.99	0.68	3	0.88
Streptomyces angustmyceticus CGS B11	C1- YMA +La	100	0.37±0.18	0.98±0.21	0.41(0.13-0.96)	8.54 (3.18-59.50)	3.02	3	0.39
Streptomyces angustmyceticus CGS B11	C2-YMA	100	-0.35±0.17	0.69±0.15	3.21(1.06-9.83)	224.76 (48.48-6,725)	1.89	3	0.59
Streptomyces angustmyceticus CGS B11	C2- YMA +La	100	-0.09±0.16	0.68±0.15	1.36 (0.37-3.93)	103.21(24.12-2,604)	0.51	3	0.92
Reference	Spinosad	120	-0.91±0.23	0.95±0.16	9.25 (4.58-18.14)	208.64 (87.77-791.71)	0.88	4	0.93

[1]Neonate (<24h old) larvae were used in artificial diet surface overlay assay. Mortality was scored after 24 h.
[2]YMA, yeast malt extract; La, Lanthanum; C1, compound 1; C2, compound 2.

The relative potency of CGS B11-C1La is more than 20 times greater than Spinosad (Table 3). The relative potency of the other compounds ranged from 2.80 to 6.06 which was not significantly different from that of Spinosad.

Table 3. Potency estimates of thin layer chromatography fractions isolated from ethyl acetate crude extracts prepared from 5-day-old fermentation broth of *Streptomyces angustmyceticus* strain CGS B11.

Treatment[1]	**Relative Potency**[2]	**Fiducial Limits (95%)**
Spinosad (reference)	1.00	0.30-3.32
CGS B11 C1	4.59	1.35-15.48
CGS B11 C2	2.80	0.82-9.41
CGS B11 C1-La (Rf1)	24.07	6.94-85.99
CGS B11 C2-La (Rf2)	6.06	1.79-20.56

[1]C1, compound 1; C2, compound 2; Rf, retention factor

It has been reported previously that REE-mediated activation of cryptic gene clusters in Actinobacteria might contribute to the discovery of novel bioactive compounds (Ochi and Hosaka 2013). This mechanism might also be responsible for the successful production of the insecticidal compound CGS B11-C1La. One possible explanation of the more than 20-fold increase in potency of CGSB11-C1La might be the presence of hydroxyl group in the pharmacophore of the molecule. The hydroxyl group might bedirectly involved in tight binding to a different target receptor site. Tight binding to receptor sight in target insect is an important factor contributing to higher insecticidal activity (Li et al. 2013).

We are not aware of any previous reports on the positive effect of Lanthanum as a chemical elicitation agent in insecticidal Actinobacteria. In another study, Lanthanum together with another rare earth element Scandium was reported to induce the production of actinorhodin in *Streptomyces coelicolor* (Tanaka et al. 2010). The bioassay results also showed that even without chemical elicitation the insecticidal compounds present in CGS B11 were of comparableinsecticidal activity with Spinosad. Aside from toxicity to ACB, CGS B11 crude extract also contained insecticidal activity to mango fruitfly, *Bactrocera philippinensis* and whitefly *Bemisia tabaci* (data not shown). The culture medium and conditions used for cultivation mighthave promoted the production of more than one insecticidal compound in the crude extract of CGS B11which gave it wider spectrum of insecticidal activity. It might be possible that the two compounds detected by TLC in CGS B11 were acting independently or in synergy. These insecticidal compounds could be further investigated for possible synergistic action with the REE-elicited insecticidal compounds to produce even more greater potency for control of ACB and other insect pests. The bioassay results observed in this study is consistent with previous observation that insecticidal compounds derived from Actinobacteria were effective against several insect pests (Kumari et al. 2014).

This is the first report of a strain (CGS B11) of *S. angustmyceticus* having insecticidal activity to insect pests. The scope of this study is limited only to insecticidal effects of Lanthanum-elicited compounds to larvae of ACB. We did not attempt to determine the insecticidal activity of the Lanthanum-elicit compounds to other insect pests. The effect of other rare earth elements was also not explored in this study. It is possible that CGS B11 when treated with other rare earth elements, singly or in combination with Lanthanum might elicit additional new insecticidal compounds with novel mode of action and/or greater potency to larvae of ACB or to other insectpests. The discovery of new insecticidal compounds from CGS B11 by various REE elicitation would be a significant development considering that the number of compounds in new classes ofinsecticides is likely small (Sparks 2013).

Insect bioassay is an important experimental tool to estimate the efficacy of insecticidal compounds. Despite the limitation in the number of insects used in the experiment, the insect mortality data fits well with the probit model as shown by insignificant $\chi 2$ value for each set ofbioassay data. The estimated slopes from the probit analysis indicated that the test insects usedfor the bioassay were homogenous. These favorable characteristics of the performed bioassay could be replicated in a wider number of test insects with calculable dependability in each case because we are following a standardized bioassay protocol for ACB in the laboratory (Hoskins and Craig 1962). Because of limitation in the amount of insecticidal compound obtained from the TLC analysis, samples from crude extracts were directly used for FTIR analysis. FTIR spectroscopy has been used directly to identify biologically active functional groups in crude samples (Sankarganesh and Joseph 2016).

Purification and structure elucidation of the promising insecticidal fractions produced by Lanthanum elicitation will be needed for establishing novelty in terms of structure and mode ofaction. The discovery of a new mode of action insecticide would be very useful for IRM, especially in the design of insecticide rotation for field application (Thompson et al. 2008).

CONCLUSION

Lanthanum elicitation is a highly effective method for the production of insecticidal compound in CGS B11. The elicited insecticidal compound had greatly improved insecticidal activity to ACB larvae. Possible synergism of the insecticidal fractions could also provide an alternative mechanism toenhance potency and at the same time lessen the cost and negative impact of synthetic insecticides. Designing a suitable formulation of the said insecticidal compounds will be required to evaluate the insecticidal potency under field conditions.

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