JOURNAL OF PURE AND APPLIED MICROBIOLOGY, FEBRUARY 2014. Vol. **8**(1), p. 701-706

# Endophytes - From Plant to Plants

**A. Garg, T. Saify, Gary Strobel, V. Kucheria, C. Prajapati, H. Kachchhava, D. Pote, B.D Patil and R. Srivastava**

RPG Life Sciences India. 2702/A GIDC Ankleshwar Gujarat, India.

(Received: 06 March 2013; accepted: 28 May 2013)

**A total of 402 endophytes were isolated from the plant *Catharanthus roseus* collected from different regions of India. Only four endophytes represented 01 – 04 mg/L peaks of alkaloid matching with the retention time of reference standards of vincristine and vinblastine. These endophytes can be further be developed to commercial scale as future source for Vinca alkaloid/s.**

**Keywords:** Endophyte, vincristine, vinblastine, *Catharanthus roseus*.

Vinca alkaloids (va) are fairly effective in combating various types of cancer, particularly Hodgkin's disease and other types of blood cancer. Catharanthus roseus is known to produce various indole alkaloids useful as an antitumor, hypotensive or antiarrhythmic agent.

Unfortunately, at the present time, the supplies of (va) Vinca Alkaloid are inadequate to meet the current or projected demands. One tonne of *Vinca rosea* leaves yields only 40 grams of (va) Vinca Alkaloid which is substantially a very low yield. Various produres documents described the synthesis of vincristine and vinblastine by coupling catharanthine and vindoline as another approach to meet the demands of the compound[1]. Other Laboratories have converted vinblastine to Vincristine by oxidation under very strict, controlled conditions[2]. Unfortunately, these prior producres also yielded small quantities of the target compounds.

Plants are commonly hosts to a multitude of microbes including parasites, symbionts, endophytes, epiphytes, and mycorrhizal fungi[3,4,5]. The production of secondary plant metabolites such as taxol may also be influenced and triggered by elicitors from microbes. Such microbes may catabolize or derivatize plant compounds.

Clearly, a microbial source of Vinca Alkaloid would be preferable if it could be easily grown and would produce Vinca alkaloid, and utilize the enormous biotechnology fermentation industry capabilities. U.S. Pat. No. 5,958,741, 5,019,504, 5,908,759, 5,916,783, 5,981,777, 6,013,493[13-18], are the various patents representing production of secondary metabolite by a Microbe.

The basis for this concept is that microbes may exist which will produce Vinca Alkaloid because of a possible genetic exchange which may have previously occurred, either between the microbe(s) or *Catharanthus roseus* plant as original source of Vinca Alkaloid. The net result would be the most desirable case of possessing one or more microbes which could be placed in fermenters to produce Vinca Alkaloid or related Vinca alkaloids

## MATERIALS AND METHODS

### Selection, Collection and identification of plant Catharanthus roseus

*C. roseus* was selectively collected from its natural habitats from eastern and western Himalayas of India. *Catharanthus roseus* was

* To whom all correspondence should be addressed.
E-mail: ankur.garg@rpgls.com

ideally selected for the study out of the eight known species of *Catharanthus*, as genus roseus is known to produce vincristine and Vinblastine. Whole plants were collected and were further subjected to isolation of endophytes[19].

**Isolation of Endophytic Microbes**

The plant material was brushed thoroughly with soap to remove surface dirt and decrease surface tension and the plant material was rinsed with tap water. Soaked for up to 5 minutes separately in alcohol (70% ethanol) and bleach (20% commercial (hypochlorite) bleach) for up to 5 minutes each (in either order). The alcohol and/or bleach soaks may were repeated if required further. The surface-sterilized plant material was then rinsed with sterile water. It was then cut into small pieces (0.5 to 1.0 cm in longest dimension) with a sterile scalpel and placed on solid water agar medium. This dual surface-sterilization technique kills contaminants more effectively than ethanol or bleach treatments singly[6,7,8].

Fungal hyphae began to grow from the plant material after two days. Pure cultures are obtained using tips of fungal hyphae as soon as they appear[10].

Isolated pure cultures were maintained on Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) for fungal cultures, while for bacterial cultures nutrient agar (NA) and nutrient broth (NB) was used. Cultures extracted with methanol after 168 Hours.( (10 ml Broth was centrifuged at 3000 RPM for 10 minutes. The supenatent was discarded and the pellet was added with 10 ml of methanol. After 5 minutes of Sonication the supernatant was filtered with 0.22 micron filter and analysed by HPLC ) using standard Pharmacopeial procedure.

**Identification of Vinca alkaloid producing Fungi**

Va are tentatively identified from in extractions of culture media using HPLC retention times of authentic standards. After confirmation of the matching peaks in chromatograms the respective standards were further spiked in the endophyte samples to confirm the presence of desired va peak in the endophyte extract.

## RESULTS

**Isolation and identification of potential Vinca alkaloid producing Endophyte**

402 endophytes were isolated from various parts of the plant as mentioned in Table - 01 and were cultured in their respective medium. Endophytes after culture in their respective

**Table 1.** Types of endophytes maintenance and production medium.

| Plant Part | No. of Endophyte | Type of Microbe | Maintenance Medium | Production medium |
|---|---|---|---|---|
| STEM | 122 | 36 (Bacterial) | NA | NB |
| | | 86 (Fungal) | PDA | PDB |
| LEAF | 84 | 20 (Bacterial) | NA | NB |
| | | 64 (Fungal) | PDA | PDB |
| ROOT | 196 | 58 (Bacterial) | NA | NB |
| | | 138 (Fungal) | PDA | PDB |
| Total | 402 Endophytes | | | |

**Table 2.** Endophytes representing peaks corresponding to Vinca alkaloids by HPLC method

| Endophyte number | Type | Harvest Time (Hrs) | Vinca alkaloid (mg/L) | | | |
|---|---|---|---|---|---|---|
| | | | Vincristine | Vinblastine | Vindoline | Catharanthine |
| RPGLS-01 | Fungal | 168 | 2.0 | Nil | Nil | Nil |
| RPGLS-02 | Bacterial | 168 | 1.0 | Nil | Nil | Nil |
| RPGLS-03 | Fungal | 168 | Nil | 4.0 | Nil | Nil |
| RPGLS-04 | Fungal | 168 | 1.0 | 2.0 | Nil | Nil |

production medium were extracted with methanol as the primary solvent. The methanolic extract was then analysed in HPLC keeping standard of Vincristine, Vinblastine, vindoline and catharanthine as control/reference standard. Of the total endophytes screened, 04 endophytes represented peaks matching with the retention time of the standard compound as mentioned in the below Table 02. The harvest hour for all the screens was kept at 144 and 168 hrs. The best results as compiled were detected at 168 Hrs.

The titer – mg/L was calculated based on the standard formula with respect to the reference compound standard area in the HPLC run.

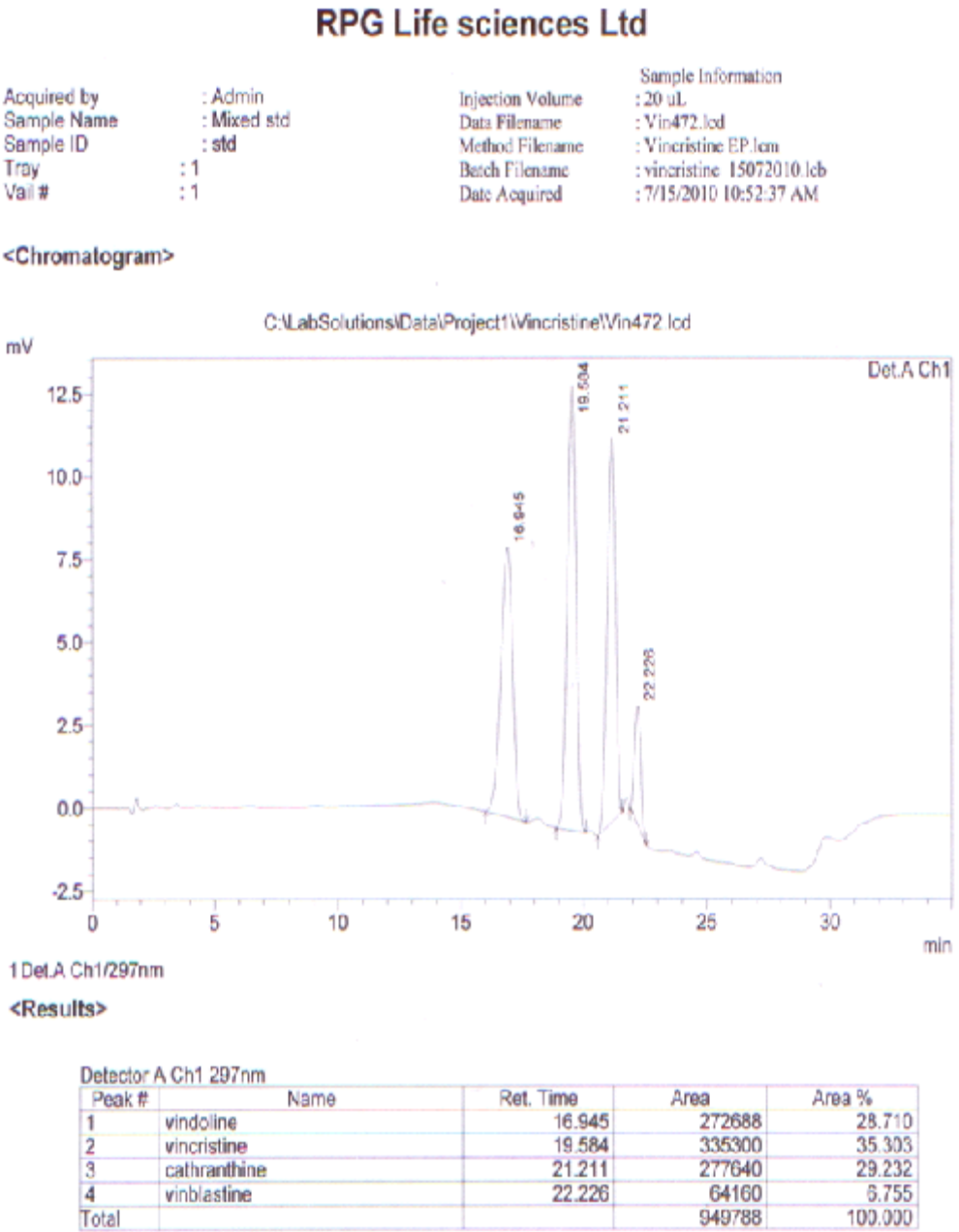



**Fig. 1(a).** Represent the retention time of reference vinca Alkaloids Standard by HPCL chromatogram

**Description of Identified Endophytes**

**Endophyte RPGLS-01**

This is a fungal endophyte and was found to contain chromatographic peak corresponding to the peak of Vincristine. The methanolic extract of the endophyte represented an activity titer of 2.0 mg/L for vincristine. Peaks of Vinblastine and other Vinca alkaloids were not found.

**Endophyte RPGLS-02**

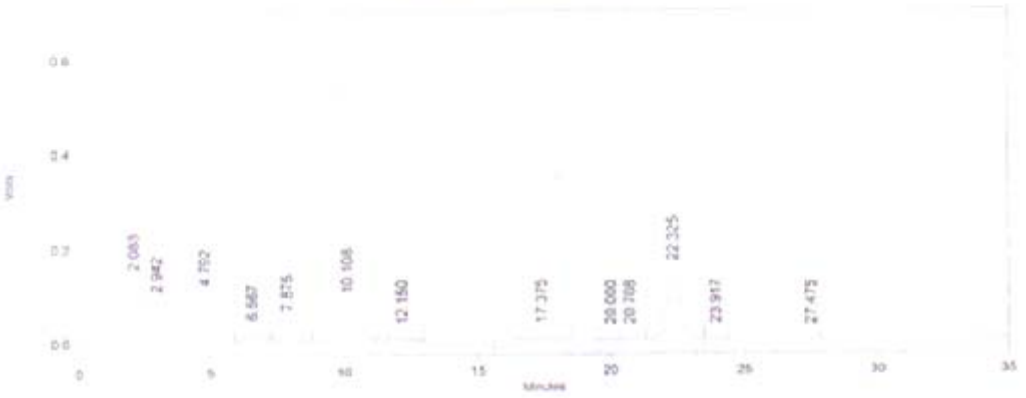
This is a bacterial endophyte and was found to contain chromatographic peak corresponding to the peak of Vincristine. The methanolic extract of the endophyte represented an activity titer of 1.0 mg/L for vincristine. Peaks of Vinblastine and other Vinca alkaloids were not found.

**RPG LIFE SCIENCES LTD.**
**R&D CENTRE, THANE**

Shimadzu CLASS-VP V6.14 SP1

Method Name :C:\CLASS-VP\Method\vincristine sulphate EP .met
Data Name :D:\HPLC\Analytical\VO 2\VIN 059
User :KIRAN
Acquired :2/10/2010 3:55:37 PM
Printed :2/10/2010 4:49:10 PM
Sample ID :VO2 /01/10 LOT-01 + Vincristine std

**Chromatogram and Report**




| Detector A (297nm) Pk # | Retention Time | Area | Area Percent |
|---|---|---|---|
| 1 | 2.08 | 2258296 | 14.26 |
| 2 | 2.94 | 1408078 | 8.89 |
| 3 | 4.79 | 2103180 | 13.28 |
| 4 | 6.57 | 370298 | 2.34 |
| 5 | 7.88 | 1065788 | 6.73 |
| 6 | 10.11 | 3407976 | 21.52 |
| 7 | 12.15 | 73099 | 0.46 |
| 8 | 17.38 | 251690 | 1.59 |
| 9 | 20.00 | 16396 | 0.10 |
| 10 | 20.71 | 16968 | 0.11 |
| 11 | 22.32 | 4799119 | 30.30 |
| 12 | 23.92 | 54508 | 0.34 |
| 13 | 27.48 | 11824 | 0.07 |
| Totals | | 15837220 | 100.00 |

**Fig. 1(b).** Represent the chromatogram of Endophyte RPGLS-03 by HPCL confirming the presence of Vinblastine peak at 22.32 retention time (Minutes)

Formulation for Titer Calculation :

Determine the average area for each standard weighing, test weighing and calculate the results in per cent purity on anhydrous & Acetone free basis as follows :

$$\% \text{ Assay} = \frac{TA \times Ws \times P \times 100 \times 100}{SA \times Wt}$$

Where,

TA = Average area of test preparation
SA = Average area of standard preparation
Ws = Weight of reference standard (mg)
Wt = Weight of sample (mg)
P = % Purity of Rapamycin reference standard.

**Endophyte RPGLS-03**

This is a fungal endophyte and was found to contain chromatographic peak corresponding to the peak of Vinblastine. The methanolic extract of the endophyte represented an activity titer of 4.0 mg/L for Vinblastine. Peaks of other Vinca alkaloids were not found.

**Endophyte RPGLS-04**

This is a fungal endophyte and showed a chromatographic peak corresponding to the peak of vinblastine. The methanolic extract of the endophyte extract showed an activity titer of 2.0 mg/L for vinblastine, along with 1.0 mg/L for vincristine, while other va not detected.

Each activity was repeated in triplicates for all endophytes. The confirmed strain was purified and preserved after lyophilization in RPG repository for maintenance and further developmental purposes.

## CONCLUSION

As apparent from the topic- PLANT (Catharanthus roseus) TO PLANTS (Industry) a commercially viable source of va is identified which could be further optimized and developed to combat the growing shortage of these drugs and also substantially reduce the price of anticancer therapy in future.

## DISCUSSIONS

Out of the 402 isolated endophytes isolates from various parts of the Catharanthus roseus plant (Table-01) only four endophytes represented peaks of va which were corresponding to the retention time of reference standards purchased from Sigma Chemicals USA. It is noticed that only vinblastine and Vincristine are found to be produced by the endophytes whereas the vindoline and catharanthine are not detected in the extracts. Endophyte RPGLS-03 extract which showed a peak at retention time of 22.32 minutes (Figure 1a and Figure 1b) corresponded with the peak of reference standard peak.

The titer – mg/L was calculated based on the standard formula with respect to the reference compound standard area in the HPLC run.

## ACKNOWLEDGEMENT

I acknowledge Dr. Gary Strobel (Professor, Montana State Univ.) for his publications and guidance.

## REFERENCES

1. Hayato Ishikawa , David A. Colby , and Dale L. Boger. Direct Coupling of Catharanthine and Vindoline to Provide Vinblastine: Total Synthesis of (+)- and ent-(“)-Vinblastine. *J. Am. Chem. Soc*.,2008: 420-421
2. Ahn SH, Duffel MW, Rosazza JP. Oxidations of vincristine catalyzed by peroxidase and ceruloplasmin. *J Nat Prod*.,1997 :**11**:1125-9.
3. Yang, X., Strobel, G.A., Stierle, A., Hess, W.M., Lee, J. and Clardy, J. A fungal endophyte - tree relationship - Phoma sp. in Taxus wallachiana. *Plant Science*, 1994: **102**:1-9.
4. Lee, J., Lobkovsky, E., Pliam, N.B., Strobel, G. and Clardy, J. Subglutinols A & B:

Immunosuppressive compounds from the endophytic fungus - Fusarium subglutinans. *J. Org. Chem*., 1995: **60**: 7076-7077.

5. Lee, J., Yang, X., Schwartz, M., Strobel, G.A. and Clardy, J., The relationship between the rarest tree in North America and an endophytic fungus. *Chemistry and Biology*, 1995: **2**:721-727.
6. Stierle, A., Stierle, D. and Strobel, G.A. The search for taxol producing microorganisms among the endophytic fungi of the Pacific yew, Taxus brevifolia. *J. Nat. Products*, 1995: **58**: 1315.
7. Strobel, G., Yang, X., Sears, J., Kramer, R., Sidhu, R.S., and Hess, W.M., Taxol from Pestalotiopsis microspora, an endophytic fungus of Taxus wallachiana. *Microbiol*, 1996: **142**: 435-440.
8. Li, J., Strobel, G.A. Harper, J., Lobkovsky, E., and Clardy, J. Cryptocin, a potent tetramic acid antimycotic from the endophytic fungus-Cryptosporiopsis quercina. *Org Lett*., 2000: **2**: 767-770.
9. Berg G & Hallmann J .Control of plant pathogenic fungi with bacterial endophytes. Microbial Root Endophytes (SchulzBJE, BoyleCJC & SieberTN, eds), 2006: pp. 53–69. Springer-Verlag, Berlin.
10. Castillo UF, Strobel GA, Ford EJ *et al*. Munumbicins, wide-spectrum antibiotics produced by Streptomyces NRRL 30562, endophytic on Kennedia nigriscans. *Microbiology*, 2002: **148**: 2675–2685.
11. De Araujo, J. M., A. C. da Silva, and J. L. Azevedo. Isolation of endophytic actinomycetes from roots and leaves of maize (Zea maysZea mays L.). *Braz. Arch. Biol. Technol*., 2000: **43**:447-451.
12. Fisher, P. J., O. Petrini, and H. M. Lappin Scott. The distribution of some fungal and bacterial endophytes in maize (Zea maysZea mays L.). *New Phytol*., 1992: **122**:299-305.
13. Stierle; Andrea (Butte, MT), Stierle; Donald (Butte, MT), Strobel; Gary (Bozeman, MT) Taxol Production By a Microbe,. *US Patent*, 1997: **5**,958,741.
14. Christen; Alice A. (Metairie, LA), Gibson; Donna M. (New Orleans, LA), Bland; John (Kenner, LA) Production of taxol or taxol-like compounds in cell culture. *US Patent*, 1989: **5**,019,504.
15. Stierle; Andrea (Butte, MT), Stierle; Donald (Butte, MT), Strobel; Gary (Bozeman, MT) Taxol production by a microbe. *US Patent*, 1999: **5**,908,759.
16. Stierle; Andrea (Butte, MT), Stierle; Donald (Butte, MT), Strobel; Gary (Bozeman, MT) Taxol production by a microbe. *US Patent*, 2000: **6**,013,49.
17. Durzan; Don J. (Davis, CA), Ventimiglia; Frank F. (Davis, CA), Recovery of taxanes from plant material. *US Patent*, 1999: **5**,981,777.
18. Stierle; Andrea (Butte, MT), Stierle; Donald (Butte, MT), Strobel; Gary (Bozeman, MT) Taxol production by a microbe. *US Patent*, 1999 *:* **5**,916,783.
19. Pham Quang Hung and K. Annapurna, Isolation and characterization of endophytic bacteria in soyabean 2004 (glycine sp.).
20. Puri SC, Verma V, Amna T, Qazi GN, Spiteller M. An endophytic fungus from Nothapodytes foetida that produces camptothecin. *J Nat Prod*., 2005 :**12** :1717-9.