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**Quorum sensing intervened bacterial signaling: Pursuit of its cognizance and repression**  
 Kayen Vaidyan, Abbas Alam Choudhury, Ramya Gurusekaran, Janarthanan Hemapriya, Selvam Vijayamand  
 Pages 235-252

[Download PDF](#) [Article preview](#)

[Short communication](#) • [Open access](#)

**Phylogenetic diversity and biotechnological potentials of marine bacteria from continental slope of eastern Arabian Sea**  
 Anilowasmi Kaber Farha, Thanasen TR, Anushi Paruthitharan, Jasatha Abdul Salam, Abdulla Mohamed Hatha  
 Pages 253-258

[Download PDF](#) [Article preview](#)

[Research article](#) • [Open access](#)

**Valorisation of chicken feathers for xanthan gum production using *Xanthomonas campestris* MO-63**  
 Hurat Ozdel, Esbi Esesen Kurbanoglu  
 Pages 259-263

[Download PDF](#) [Article preview](#)

[Research article](#) • [Open access](#)

**Biolytic extraction of poly(3-hydroxybutyrate) from *Bacillus megaterium* T13 using the lytic enzymes of *Streptomyces albus* T1a1**  
 Nafsa Inani, Suresh Thapa, Shweta Shrivastava  
 Pages 265-273

[Download PDF](#) [Article preview](#)

[Research article](#) • [Open access](#)

**Purification and characterization of alkaline soda-bleach stable protease from *Bacillus* sp. APP-07 isolated from Lauromat soil**  
 I.K. Shukla, P.P. Dixit, T.M. Shukla  
 Pages 273-279

[Download PDF](#) [Article preview](#)

[Research article](#) • [Open access](#)

**Improvement of cellulose degradation by cloning of endo- $\beta$ -1, 3-1, 4 glucanase (*lg1c*) gene from *Bacillus subtilis* BTN7A strains**  
 Wafaa K. Hegazy, Mohamed S. Abdel-Salam, Achar A. Hussain, Huda H. Abu-Ghalla, Safa S. Hafez  
 Pages 281-289

[Download PDF](#) [Article preview](#)

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**Antibacterial activity of soil bacteria isolated from Kochi, India and their molecular identification**  
 Datta Gopin, Dhanraj Subramaniam, Ganesaprasadam Arany Raj, Kathiravan Basilan  
 Pages 287-294

[Download PDF](#) [Article preview](#)

[Research article](#) • [Open access](#)

**Purification and characterization of alkaline protease with novel properties from *Bacillus cereus* strain S8**  
 E.K.M. Lailahani, D. Mura Kurnia, K.P.J. Hernalatha  
 Pages 295-304

[Download PDF](#) [Article preview](#)

[Research article](#) • [Open access](#)

**Enhancement of nematocidal potential through cloning and expression of *chitinase* gene from *Bacillus subtilis* subsp. *subtilis* BTN7A strain**  
 Mohamed S. Abdel-Salam, Huda H. Amey, Abdelrahman S.M. Kasab, Ahmed E.A. Mahgoub, Usama S. Elshamy  
 Pages 305-312

[Download PDF](#) [Article preview](#)

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**Biodegradation of feather waste by keratinase produced from newly isolated *Bacillus licheniformis* ALW1**  
 Asa H. Abdel-Fattah, Maroudiah S. El-Garni, Shaim A. Ismail, Mohamed A. Elwan, Amal M. Hakeem  
 Pages 311-318





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**Biodegradation of feather waste by keratinase produced from newly isolated *Bacillus licheniformis* ALW1**  
Aza M. Abdel-Fattah, Mamdouh S. El-Garni, Sofan A. Ismail, Mohamed A. Elwan, Amal M. Hashem  
Pages 311-318  
[Download PDF](#) [Article preview](#)
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**Study on the potential of cold-active lipases from psychrotrophic fungi for detergent formulation**  
Srinjay Sakay, Deepak Chouhan  
Pages 319-325  
[Download PDF](#) [Article preview](#)
- [Research article](#) • [Open access](#)  
**Optimization of novel halophilic lipase production by *Fusarium solani* strain NFCCCL-4084 using palm oil mill effluent**  
Kiptoo Geoffry, Rajeshwari N. Achur  
Pages 327-334  
[Download PDF](#) [Article preview](#)
- [Research article](#) • [Open access](#)  
**Cloning and expression of MPT83 gene from *Mycobacterium tuberculosis* in *E. coli* BL21 as vaccine candidate of tuberculosis: A preliminary study**  
Aliyar Ahmad, Rozana Agus, Muh. Nazam Muzt, Rodiana Nizati, ... Masugi Maruyama  
Pages 335-340  
[Download PDF](#) [Article preview](#)
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**Immobilization of thermostable  $\alpha$ -amylase from mutant thermophilic *Aspergillus tamarii*-U4 using kaolin clay and its application in inulin hydrolysis**  
Emmanuel O. Garuba, Abiodun, A. Omluke  
Pages 341-346  
[Download PDF](#) [Article preview](#)
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**High level expression and purification of recombinant founder growth hormone in *E. coli***  
Tae-Jin Choi, Taejeong Toh Geleda  
Pages 347-355  
[Download PDF](#) [Article preview](#)
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**Screening of potential probiotic lactic acid bacteria and production of amylase and its partial purification**  
Tallapragada Padmanathi, Rajeswaru Shargan, Purushothama Rao Priyanka, Naiga Ranganath Niranjan, Pogalal Venobhadrapa Perma  
Pages 357-362  
[Download PDF](#) [Article preview](#)
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**Partial purification and characterization of  $\alpha$ -amylase produced from *Bacillus* sp.**  
R. Ramapriya, A. Thirumangalakudi, T. Sathishkumar, D.R. Manikandan  
Pages 363-367  
[Download PDF](#) [Article preview](#)
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**Effect of vitamins and cell constructions on the activity of microbial fuel cell battery**  
Dera Z. Khater, E.M. El-Khatib, Rabey YA. Hassan  
Pages 369-373  
[Download PDF](#) [Article preview](#)
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**Decolorization of Textile Reactive Dyes by Bacterial Monoculture and Consortium Screened from Textile Dyeing Effluent**  
Md. Enayul Karim, Karik Dhar, Md. Tawhid Hossain  
Pages 375-380  
[Download PDF](#) [Article preview](#)
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**Optimization of quorum quenching mediated bacterial attenuation of *Solanum torvum* root extract by response surface modelling through Box-Behnken approach**  
Rajeev Vadakhan, Selang Vijayanand, Abbas Alam Choudhury, Ranya Gunasekaran, Jayantharam Hemapriya  
Pages 381-386  
[Download PDF](#) [Article preview](#)
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**Isolation and characterization of *Bacillus* sp. strain BC01 from soil displaying potent antagonistic activity against plant and fish pathogenic fungi and bacteria**  
Md Javed Fayal, Azra Khawar Lia  
Pages 387-392  
[Download PDF](#) [Article preview](#)
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**Expression of *Leptospira* membrane proteins Signal Peptidase (SP) and *Leptospira* Endostatin like A (Lm A) in BL-21(DE3) is toxic to the host cells**  
Paullina K. Satheshkumar, Prasanna V. Anu, Mohamed I. Jaseeda, Madhupratim G. Mahanta, ... Perumona R. Subhakaran  
Pages 393-398  
[Download PDF](#) [Article preview](#)

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**Scedermus obliquus: Antioxidant and antiviral activity of proteins hydrolyzed by three enzymes**  
 Abd El-Moneem M.R. Abof, Gamal S. El Baroty, Farouk K. El Baz, Hanaa H. Abd El Esky, Saha A. Murrad  
 Pages 399-408  
[Download PDF](#) [Article preview](#)
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**Statistical optimization of crude oil bio-degradation by a local marine bacterium isolate *Pseudomonas* sp. sp48**  
 Seha Fereq, Nadia A. Selman, Yasser R. Abdel-Fattah  
 Pages 409-420  
[Download PDF](#) [Article preview](#)
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**Influence of bioprocess variables on the production of extracellular chitinase under submerged fermentation by *Streptomyces pratensis* strain KLSL55**  
 A. Sivadas, R. Lingappa, Divakar Mahesh  
 Pages 421-426  
[Download PDF](#) [Article preview](#)
- Medical Biotechnology**
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**Recent advances in stem cells therapy: A focus on cancer, Parkinson's and Alzheimer's**  
 Dalia Flaifel, Mai Abel Rahmoon, Abdelrahman AlDida, Mostafa Naar, ... Sherif F. El-Khamisy  
 Pages 427-432  
[Download PDF](#) [Article preview](#)
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**In vitro differentiation of human multilineage differentiating stress-enduring (Muse) cells into insulin producing cells**  
 Ali M. Fouad, Mahmoud M. Gabr, Elsayed E. Akelahady, Mahmoud M. Zakaria, ... Ayman F. Refae  
 Pages 433-440  
[Download PDF](#) [Article preview](#)
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**Development and evaluation of latex agglutination test coating with recombinant antigen, LipL32 for serodiagnosis of human leptospirosis**  
 Kotchakorn Thongsukhaeng, Rangsit Boonyom  
 Pages 441-446  
[Download PDF](#) [Article preview](#)
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**FNME – A gene-gene parallel network module extraction method**  
 Bkash Jainel, Kumar Utkarsh, O.K. Shattacharyya  
 Pages 447-457  
[Download PDF](#) [Article preview](#)
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**Expression, purification and biological characterisation of recombinant human irisin (12.5 kDa)**  
 Kalpana Parati, Venkata Ramimadhy Narala, Vyjayanth R. Naresimha, Madhavi Derangula, ... Somesha Yegorappa  
 Pages 459-466  
[Download PDF](#) [Article preview](#)
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**Increased level of B cell differentiation factor in systemic lupus erythematosus patients**  
 Hala Zaki Raslan, Hiba Sibai, Salwa Refat El- Zayat, Hagar Hassan, Mahdiya El- Kassaby  
 Pages 467-471  
[Download PDF](#) [Article preview](#)
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**Healthcare-associated (HA) and community-associated (CA) methicillin resistant *Staphylococcus aureus* (MRSA) in Bangladesh – Source, diagnosis and treatment**  
 Md. Anwar Khasru Parvez, Rabeya Nahar Ferdous, Md. Shahetar Rahman, Sehidul Islam  
 Pages 473-478  
[Download PDF](#) [Article preview](#)
- [Research article](#) • [Open access](#)  
**Assessment of Ki-67 as a potential biomarker in patients with breast cancer**  
 Hala Mohamed Ragab, Nerwana Samy, Mia APS, Nabila Abd El Hakeoud, Hababallah Mohamed Sheaban  
 Pages 479-484  
[Download PDF](#) [Article preview](#)
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**Kolaviron and selenium reduce hydrogen peroxide-induced alterations of the inflammatory response**  
 Tahmineh Oskola  
 Pages 485-490  
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**Feline panleukopenia viral infection in cats: Application of some molecular methods used for its diagnosis**  
Romana A. Awad, Wagdy K.B. Khalil, Ashraf G. Attalah  
Pages 493-497  
[Download PDF](#) [Article preview](#) 
  
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**Buffalo species identification and delineation using genetic barcoding markers**  
Amal Ahmed Mohamed Hassan, Enas Aly Salabel, Hanaa Abdel Sadak Oraby, Samy Amour Darwish  
Pages 499-505  
[Download PDF](#) [Article preview](#) 
  
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**Detection of myostatin gene MSTN in some goat breeds (*Capra hircus*)**  
Y.A. Dawidar, M.A. El-Sayed, Aly M. Elnefy, Hytham E. Shoura  
Pages 507-512  
[Download PDF](#) [Article preview](#) 
  
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**Five BoLA-DRE3 genotypes detected in Egyptian buffalo infected with Foot and Mouth disease virus serotype O**  
Othman E. Othman, Muhammad G. Khodairy, Ayman H. El-Diabi, Hussein A. Hussein  
Pages 513-518  
[Download PDF](#) [Article preview](#) 

## Nano-Biotechnology

- [Research article](#) • [Open access](#)  
**Cytogenetic effects of silver and gold nanoparticles on *Allium cepa* roots**  
Priyanka Debnath, Anghusip Mondal, Anrita Hajra, Chittaranjan Das, Naba Kumar Mondal  
Pages 519-528  
[Download PDF](#) [Article preview](#) 
  
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**Synthesis of silver nanoparticles by *Bacillus clausii* and computational profiling of nitrate reductase enzyme involved in production**  
Kael Mukherjee, Rashmi Gupta, Gourav Kumar, Sanjay Kumar, L. Padmini Padmanabhan  
Pages 527-538  
[Download PDF](#) [Article preview](#) 

## Plant Biotechnology

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**Transgenic approaches for genetic improvement in groundnut (*Arachis hypogaea* L.) against major biotic and abiotic stress factors**  
Srilat Gantait, Suvendu Mondal  
Pages 537-544  
[Download PDF](#) [Article preview](#) 
  
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**In vitro biotechnological advancements in Malabar nut (*Adhatoda vasica* Nees): Achievements, status and prospects**  
Srilat Gantait, Jitendra Patra  
Pages 545-552  
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**Elevated carotenoids in staple crops: The biosynthesis, challenges and measures for target delivery**  
Adebanjo Ayobamidele Badajo  
Pages 553-562  
[Download PDF](#) [Article preview](#) 
  
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**In vitro culture, transformation and genetic fidelity of Milk Thistle**  
M.K. Redy, M.M. Salim, M.A. Matar  
Pages 563-572  
[Download PDF](#) [Article preview](#) 
  
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**Cloning, transformation and expression of cell cycle-associated protein kinase OsWee1 in indica rice (*Oryza sativa* L.)**  
Frengly H.H. Prasetyo, Bambang Sugiharto, Natty Ermasari  
Pages 573-579  
[Download PDF](#) [Article preview](#) 
  
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**Optimization of indole acetic acid production by isolated bacteria from *Stevia rebaudiana* rhizosphere and its effects on plant growth**  
Sheela Chandra, Razim Aslan, Madhumba Kumar  
Pages 581-586  
[Download PDF](#) [Article preview](#) 

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**Plant regeneration, developmental pattern and genetic fidelity of somatic embryogenesis derived *Musa* spp.**  
 Natarajan Nandhakumar, Kish Kumar, Duraidaganga Sudhakar, C. Sorianathasundaram  
 Pages 587-598  
[Download PDF](#) [Article preview](#)
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**Population structure, morphological and genetic diversity within and among melon (*Cucumis melo* L.) landraces in Iran**  
 Masoud Maleki, Abdolali Shejarian, Sajad Rezaei Morfared  
 Pages 599-606  
[Download PDF](#) [Article preview](#)
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**Influence of cold pretreatment on shoot regeneration from callus in date palm (*Phoenix dactyloides* L.) cv. 'Barhee'**  
 Ahmad Magi Wuhaed Al-Mayah, Abdulminem Hussein Ali, Hussein J. Sharaf  
 Pages 607-612  
[Download PDF](#) [Article preview](#)
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**Screening of plant growth promoting traits in heavy metals resistant bacteria: Prospects in phytoremediation**  
 N. Tomy, N. Tahir Joutay, H. Sayal, A. Kouchou, ... N. El Ghaetouli  
 Pages 613-619  
[Download PDF](#) [Article preview](#)
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**Phytochemical analysis, antioxidant and antimicrobial activity of wild and *in vitro* derived plants of *Crotophia thurstonii* Hook – An endemic species from Western Ghats, India**  
 S. Muthukrishnan, T. Senthil Kumar, A. Gangaprasad, F. Wagg, M.V. Rao  
 Pages 621-630  
[Download PDF](#) [Article preview](#)
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**Molecular diversity of internal transcribed spacer among the monoconidial isolates of *Magnaporthe oryzae* isolated from rice in Southern Karnataka, India**  
 D. Jagadeesh, M.K. Prasanna Kumar, R. Chandrakanth, N.S. Devaki  
 Pages 631-638  
[Download PDF](#) [Article preview](#)
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**Production of biomass and flavonoid of *Cynura procumbens* (Lour.) Merr shoots culture in temporary immersion system**  
 Ayu Dewi Pramita, Alinda Novi Kristanti, Sugiharto, Ety Seti Widi Utami, Yosephine Sri Wulan Manuhara  
 Pages 639-643  
[Download PDF](#) [Article preview](#)
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**Callus mediated shoot organogenesis and regeneration of cytologically stable plants of *Labeourea revoluta*: An ethnomedicinal plant with promising antimicrobial potency**  
 Sr. Moqammel Haque, Anjit Chakraborty, Binayit Ghosh  
 Pages 645-651  
[Download PDF](#) [Article preview](#)
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**Evaluation of the alleviative role of *Chlorella vulgaris* and *Spirulina platensis* extract against ovarian dysfunctions induced by monosodium glutamate in mice**  
 Sakina H Abdel-Karem, Heba A.M. Abd El-Kader, Fatem M. Ibrahim, Hafiza A Sharaif, Aida I. El maissy  
 Pages 653-660  
[Download PDF](#) [Article preview](#)
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**Assessment of genetic diversity in *Salvidora persica* L. based on inter simple sequence repeat (ISSR) genetic marker**  
 Mohammad Akad, Morfared, Dawood Samsampour, Ghulam Reza Shari-E-Sirhi, Fatemeh Sadeghi  
 Pages 661-667  
[Download PDF](#) [Article preview](#)
- [Research article](#) • [Open access](#)  
**Micropropagation protocol for *Astigonon leptopus* an important ornamental and medicinal plant**  
 Zerra Fausta Ghareeb, Lubna S. Taha  
 Pages 669-675  
[Download PDF](#) [Article preview](#)
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**Total phenolic and flavonoid contents and antioxidant activity of ginger (*Zingiber officinale* Rosc.) rhizome, callus and callus treated with some elicitors**  
 Amratar Mohammed Ahmed Ali, Mawhib El-Emin Mohamed El-Rour, Sakina Mohamed Yag  
 Pages 677-682  
[Download PDF](#) [Article preview](#)

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**Physiological and molecular studies on the effect of gamma radiation in fenugreek (*Trigonella foenum-graecum* L.) plants**  
Rania Somy Hanafy, Samia Ageeb AlMadhou  
Pages 683-692  
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**Rice straw fermentation by *Schizophyllum commune* ARC-11 to produce high level of xylanase for its application in pre-bleaching**  
Anshu Gautam, Anil Kumar, Amit Kumar Bhatti, Dharm Dutt  
Pages 693-701  
[Download PDF](#) [Article preview](#)

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***Agrobacterium tumefaciens*-mediated transformation of *Dendrobium laniifera* J.J.Sim: An important medicinal orchid**  
Edy Seti Widhi Utami, Susanto Hariyanto, Yesshira Sri Wulan Manuhara  
Pages 703-709  
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[Short communication](#) [Open access](#)  
**Screening of anti-inflammatory phytochemicals from *Croton alstonii* leaf extracts and its validation by in silico modeling**  
Rathinavel Thirumalaikamby, Subramanian Ananthi, Govarthanan Muthusamy  
Pages 711-718  
[Download PDF](#) [Article preview](#)

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**In silico structural and functional modelling of Antifreeze protein (AFP) sequences of Ocean pout (*Zoarces americanus*, Bloch & Schneider 1801)**  
Manojit Bhattacharya, Arpita Hota, Anjita Kar, Deep Sanjay Chini, ... Sasmita Kumar Das  
Pages 721-730  
[Download PDF](#) [Article preview](#)

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**In silico structural homology modeling of nifA protein of rhizobial strains in selective legume plants**  
Sudam D.V. Satyanarayana, M.S.R. Krishna, Firdi Fauzi Kurnia, Sriela Jaenady  
Pages 731-737  
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Zehra Aminfar, Masoud Tohidfar  
Pages 738-747  
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**In silico studies on bacterial xylanase enzyme: Structural and functional insight**  
Bhramar Dutta, Aparna Samerjes, Priyanka Chakraborty, Rajit Sandopahyay  
Pages 749-758  
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**In silico thermodynamic stability of mammalian adaptation and virulence determinants in polymerase complex proteins of H9N2 virus**  
Ziaab Mosaad, Abdelaziz Arafa, Hussein A. Hussein, Mohamed A. Shalaby  
Pages 757-767  
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**Interaction of rs316019 variants of SLC22A2 with metformin and other drugs- an in silico analysis**  
Abu Ashfaqur Sayib, Tasmin Islam, Nilanjana Paul, Sabina Yasmin  
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## Original Article

# *Agrobacterium tumefaciens*-mediated transformation of *Dendrobium lasianthera* J.J.Sm: An important medicinal orchid

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Transformation

## ABSTRACT

A protocol for genetic transformation mediated by *Agrobacterium tumefaciens* and production of transgenic *Dendrobium lasianthera* has been developed for the first time. The 8-week-old protocorm explants were used as target of transformation with *Agrobacterium tumefaciens* strain LBA4404 carrying plasmid pG35SKNAT1. Several parameters such as infection period, *Agrobacterium* density, concentration of acetosyringone, and co-cultivation period were evaluated for the transformation efficiency. The data were analyzed using one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) with  $p < 0.05$ . Subsequently, KNAT1 gene expression was confirmed by polymerase chain reaction (PCR) analysis. The highest efficiency of transformation (70%) obtained from protocorm explants infected with *Agrobacterium* culture was at the OD<sub>600</sub> concentration of 0.6 for 30 min, and co-cultivated with acetosyringone 100  $\mu$ M for 5 days. The results of confirmation by PCR analysis show that the KNAT1 gene has been integrated and expressed in the genome of *Dendrobium lasianthera* transgenic.

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## 1. Introduction

Currently, orchid has become a significantly commercial commodity in Indonesia. Despite being a major part of cut flower industry, orchid specifically genus *Dendrobium* has been known as traditional medicine. In fact, traditional medicines sourced from orchid have long been circulated in China [1]. Multiple bibenzyls secondary metabolite, fluorenones and gigantol have been isolated from *Dendrobium nobile* which has a higher antioxidant activity than vitamin C [2]. Extracts from leaf, stem, root and pseudobulb of *Dendrobium crumenatum* have an anti-microbial activity [3]. New compounds of dendroside D, dendroside E, dendroside F and dendroside G have been discovered in *Dendrobium nobile* and indicated immunomodulatory activity [4]. One of orchid's species in Indonesia that has anticancer activity is *Dendrobium lasianthera* J. J.Sm.

Three vegetative organs (root, stem and leaf) of *D. lasianthera* J.J. Sm, are toxic and have anticancer activity, however, the most toxic organ with the highest breast anticancer activity T47D is stem with

LC50 ( $\mu$ g/mL) =  $117 \pm 6.35$ . Owing to its notable potential of becoming raw material for medicine and producing cut flowers, *Dendrobium lasianthera* is of high economic value and is promising to be cultivated.

The main problems in the development of orchid plant to be used as raw material for medicine are: the technique mass propagation is relatively difficult, too long vegetative phase in its life cycle (1–2 years), and genetic stability of the plant. To increase orchid production, genetic engineering is applied by inserting foreign gene into genome of *Dendrobium lasianthera* mediated by *Agrobacterium tumefaciens*.

The insertion of foreign genes into the genome of plants mediated by *Agrobacterium tumefaciens* is an effective and reproducible method and has been successfully applied to various plants such as *Artemisia carvifolia* [5], *Woodfordia fruticosa* [6], *Solanum trilobatum* [7], *Withania somnifera* [8], *Vanda kasem's* [9], and *Erycina pusilla* [10].

The genetic transformation by inserting *KNAT1* (*KNOTTED1* like *Arabidopsis thaliana*) gene into *Phalaenopsis amabilis* Blume has been done by Semiarti et al. which resulted in the formation of multiple shoots from one protocorm [11]. Recently, more success of genetic transformation in medicinal plants has been reported [7,12–13]. However, gene transformation of *KNAT1* into

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*D. lasianthera* protocorm mediated by *A. tumefaciens* has not been found yet.

*KNAT1* is a group of first class KNOX gene which is successfully isolated and characterized from *Arabidopsis thaliana* and functions to organize formation, development, and maintenance of meristem in stem tip to keep the cells meristematic. Over-expression of *KNAT1* in *Arabidopsis* causes formation of adventitious shoots on both sides of leaf [14].

The success of genetic transformation mediated by *A. tumefaciens* was influenced by several factors. The factors are pre-incubation, *Agrobacterium* density, *Agrobacterium* strain, infection period, acetosyringone concentration, co-cultivation period, type and concentration of antibiotic to eliminate *Agrobacterium*.

In the present study, the effect of infection period, bacterial density, concentration of acetosyringone (AS), and co-cultivation period in the modified Vacin and Went [15] medium were examined for the transformation efficiency.

## 2. Materials and methods

### 2.1. Plant materials, *Agrobacterium tumefaciens* strain and construction used for transformation

Healthy 8-week-old protocorm (Fig. 5B) from *Dendrobium lasianthera* were used as the explants for *Agrobacterium*-mediated genetic transformation.

*Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pG35SKNAT1 used for transformation was kindly given by Dr. Endang Semiarti from Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia. The T-DNA of pG35SKNAT1 contained neomycin phosphotransferase (NPTII) gene under the control of 35S cauliflower mosaic virus (CaMV) promoter (Fig. 1). Bacteria cultures were maintained at  $-80^{\circ}\text{C}$  for long term storage in 70% (v/v) glycerol.

### 2.2. Sensitivity test of protocorm to kanamycin

To identify the effective concentration of kanamycin as an agent of selection, protocorms were cultured on medium VW + 30 g/L sucrose + 2 g/L peptone + 0.5 mg/L benzyladenine + 1 mg/L thidiazuron containing different concentration of kanamycin (0, 25, 50, 75, 100 mg/L). Ten protocorms were used for each treatment, and the experiment was repeated three times. Cultures were incubated at  $25 \pm 2^{\circ}\text{C}$  under 16-h light/8-h dark photoperiod. Protocorm was sub-cultured to similar medium every three weeks for nine consecutive weeks. Observation was conducted in the ninth week to see protocorm sensitivity toward kanamycin. Protocorm was considered survived if the protocorm stayed green.

### 2.3. Suspension culture of *A. Tumefaciens*

One colony of *A. tumefaciens* strain LBA4404 harboring plasmid pG35SKNAT1 was inoculated into 10 mL of liquid LB medium with 100 mg/L kanamycin. The *A. tumefaciens* cultures were grown in

shaking culture at 150 rpm for 18–20 h at  $28 \pm 2^{\circ}\text{C}$ . Two mL suspension of *A. tumefaciens* was measured for its optical density of 0.8 ( $\text{OD}_{600\text{nm}} = 0.8$ ). Bacterial cells were collected using centrifugation at 6000 rpm for 5 min at  $4^{\circ}\text{C}$ . Supernatant was removed, added 2 mL of VW medium, vortexed, and re-suspended in 20 mL of VW medium.

### 2.4. Optimization the factors influencing the transformation efficiency

During the transformation of *D. lasianthera* mediated by *A. tumefaciens*, some factors influencing transformation efficiency were evaluated, they were bacterial density ( $\text{OD}_{600\text{nm}}$  at 0.2, 0.4, 0.6, 0.8, and 1.0), co-cultivation period (1, 2, 3, 4, and 5 days), acetosyringone concentration (0, 50, 100, 150, and 200  $\mu\text{M}$ ), and infection period (10, 20, 30, 40, and 50 min). In this study, factors that have been investigated and optimized through research and have showed the best results will be used in future research. First, we evaluated bacterial density with co-cultivation time on the third day, acetosyringone concentration 50  $\mu\text{M}$ , and infection period at 20 min. Second, we evaluated co-cultivation period with bacterial density  $\text{OD}_{600\text{nm}}$  at 0.6, acetosyringone concentration 50  $\mu\text{M}$ , and infection period at 20 min. Third, we evaluated acetosyringone concentration with bacterial density  $\text{OD}_{600\text{nm}}$  at 0.6, co-cultivation time at 5th day, and infection period 20 min. Finally, we evaluated infection period with bacterial density  $\text{OD}_{600}$  at 0.6, co-cultivation time on the fifth day, and acetosyringone concentration 100  $\mu\text{M}$ . Twenty five protocorms were used for each treatment, and the experiment was repeated four times. Kanamycin-resistant protocorm was collected after being cultured for 2 months. Transformation efficiency was determined by following formula: the amount of kanamycin-resistant protocorm is divided by the total amount of cultured protocorm x 100%.

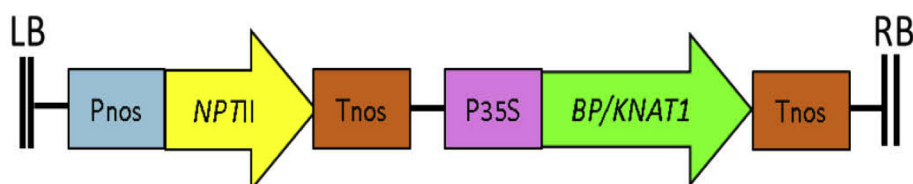
### 2.5. Transformation and regeneration

#### 2.5.1. Infection and co-cultivation

Protocorm was infected with 2 mL suspension of *A. tumefaciens* in 20 mL liquid IM medium of  $\text{OD}_{600\text{nm}}$  at 0.6 (Table 1) and shook at 100 rpm for 30 min. Next, protocorm was air dried in sterile filter paper to decrease bacterial suspension liquid. Protocorm was moved to 20 mL CCM medium (Table 1) in sterile 5 cm petridish. The plates were sealed with parafilm and kept in a dark room at  $25 \pm 1^{\circ}\text{C}$  for co-cultivation for 5 days.

#### 2.5.2. Selection and shoot induction

After co-cultivation, protocorms that have been infected were washed with sterilized aquadest three times, then air dried on sterile filter paper. Protocorm was cultured on selection medium (Table 1). The plates were then kept with a photoperiod of 16 h light/8h dark for 2 months. Next, protocorm was transferred to a sterile petridish which contained 20 mL of shoot induction medium and cultured for 3 months to distinguish kanamycin-resistant shoots. Culture was kept in the same condition as previously explained. The parameters of transformation was calculated



**Fig. 1.** Structure of the T-DNA pG35SKNAT1. BP/KNAT1 gene (1200 bp) under the control of the 35S promoter of cauliflower mosaic virus (CaMV); LB = Left Border; Pnos = promoter of the nopaline synthase gene; NPTII = neomycin phosphotransferase gene; Tnos = polyadenylation site of the nopaline synthase gene; P35S = 35S promoter of CaMV; RB = Right Border.

**Table 1**

List of medium used in the study.

Culture medium	Composition
Germination medium (GM)	VW medium + 3 g/L peptone <sup>a</sup> + 30 g/L sucrose <sup>b</sup>
Infection medium (IM)	VW medium + 100 $\mu$ M acetosyringone <sup>c</sup>
Co-cultivation medium (CCM)	VW medium + 100 $\mu$ M acetosyringone <sup>c</sup> + 30 g/L sucrose <sup>b</sup> + 0.5 mg/L thiazuron <sup>c</sup> + 0.5 mg/L benzyladenine <sup>c</sup>
Selection medium (SM)	VW medium + 500 mg/L cefotaxime <sup>c</sup> + 100 mg/L kanamycin <sup>d</sup> + 30 g/L sucrose <sup>b</sup> + 0.5 mg/L thiazuron <sup>c</sup> + 0.5 mg/L benzyladenine <sup>c</sup>
Shoot induction medium (SIM)	VW medium + 500 mg/L cefotaxime <sup>c</sup> + 100 mg/L kanamycin <sup>d</sup> + 30 g/L sucrose <sup>b</sup> + 0.5 mg/L thiazuron <sup>c</sup> + 0.5 mg/L naphthalene acetic acid <sup>b</sup> + 0.5 mg/L gibberelic acid <sup>c</sup>
Root induction medium (RIM)	VW medium + 100 mg/L kanamycin <sup>d</sup> + 30 g/L sucrose <sup>b</sup> + 0.5 mg/L indole acetic acid <sup>d</sup>

<sup>a</sup> HIMEDIA Laboratories, LBS Marg, Mumbai India.<sup>b</sup> Merck, Darmstadt, Germany.<sup>c</sup> Phyto Technology Laboratories, Shawnee Mission, United States.<sup>d</sup> Sigma-Aldrich, St. Louis, Missouri, United States.**Table 2**Summary of transformation mediated by *A. tumefaciens* of *D. lasianthera* in nine months.

Experiment	Total protocorms	No. of shoot $\geq$ 1 cm long	No. of transgenic plants <sup>b</sup>	Transformation of efficiency (%) <sup>c</sup>
Transformation	50	39	35	70
Wild type <sup>a</sup>	50	50	0	0

<sup>a</sup> Wild type: protocorms were not infected with *A. tumefaciens* and cultured on medium without kanamycin.<sup>b</sup> Transgenic plants were confirmed by positive PCR.<sup>c</sup> Transformation efficiency was calculated by number of no of transgenic divided by total protocorms  $\times$  100%.

as the percent protocorms showing shoot regenerating on selection medium (Table 2) and presence of transgene has been validated by polymerase chain reaction (PCR).

### 2.5.3. Root induction and plantlet acclimatization

Kanamycin-resistant shoots with  $\geq$  1 cm length were cultured individually on RIM medium (Table 1) for 3 months for root induction. All the cultures were maintained at  $23 \pm 1$  °C under a 16 h-light and 8 h-dark photoperiod. Following *D. lasianthera* plantlets with 3–4 leaves, bearing 3–5 roots (approximately 2–4 cm in height) were removed from the culture tube and rinsed with tap water to wipe off the agar and transplanted to plastic pots loaded a mixture of coconut fiber and sphagnum moss (3:1 v/v). Potted plants were grown in the greenhouse under 30–40% natural light and sprayed two times a day for acclimatization.

### 2.6. Plant DNA isolation and confirmation of putative transgenic using polymerase chain reaction analysis

DNA of plant genom was isolated using DNA extraction kit (Genomic DNA Minikit Plant, Geneaid, United States), following manufacturer's protocol. Genomic DNA from the fresh shoots (100 mg) of putative transgenic and non-transgenic *D. lasianthera* plants were examined by PCR amplification for the presence of *Knat1* gene. The oligonucleotide primers for *Knat1* gene were "forward": 5' CTT CCT AAA GAA GCA CGG CAG 3' and "reverse" 5' CCA GTG ACG CTT TCT TTG GTT 3'. These primers were expected to produce 1200 bp. PCR amplification was done using following program order: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 30 s, and followed by post extension for 7 min at 72 °C. The PCR products were analyzed by electrophoresis gel in 1% (w/v) agarose gels and viewed under UV transilluminator.

### 2.7. Experimental design and statistical analysis

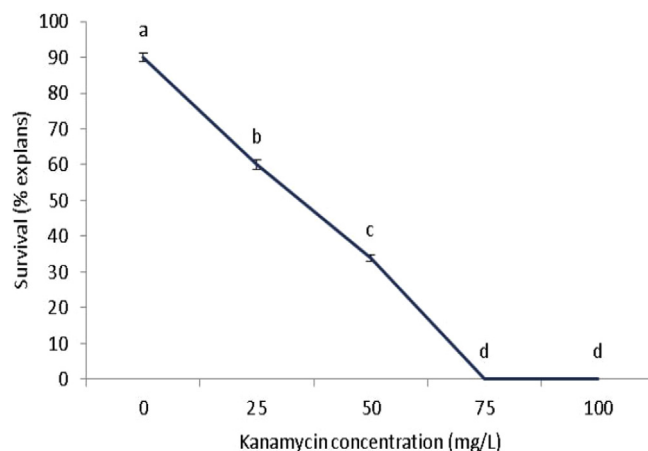
The experiment was arranged in Completely Randomized Design (CRD). The data was analyzed by one way analysis of variance (ANOVA) with SPSS (Version 20), and means of differences

among treatment were examined using Duncan's multiple range test (DMRT) at  $p < 0.05$  [16].

## 3. Results

### 3.1. Kanamycin sensitivity

Sensitivity test of *protocorm* toward kanamycin as an agent of selection in this study had been done with concentration of 0, 25, 50, 75, and 100 mg/L. The experimental results (Fig. 2) showed that kanamycin presence in medium causes significant toxicity to *protocorm* and declining survival response. A survival response of 90% was noticed on medium without kanamycin (control), higher kanamycin concentration caused a more significant decrease toward survival response that was 60% of survival response on kanamycin 25 mg/L and 34% of survival response on kanamycin 50 mg/L. The cultured *protocorm* on medium which contained kanamycin 75 mg/L and 100 mg/L produced dead *protocorm*, hence the survival response was 0%. This indicated that in those concentrations *protocorm* could not develop.



**Fig. 2.** The influence of kanamycin toward VW medium on survival *protocorm* explants. The data was recorded after 9 weeks of culture.

### 3.2. Optimization of factors influencing transformation efficiency

#### 3.2.1. Effects of bacterial density on the transformation efficiency of *D. Lasianthera*

In this study, we evaluated the influence of different bacterial density to transformation efficiency. The suspension culture of the *Agrobacterium* with OD<sub>600nm</sub> at 0.6 produced the highest transformation efficiency (67% ± 1.2), followed by 0.8 (59% ± 0.7), 0.4 (55% ± 2.1), 1.0 (52% ± 1.5), and 0.2 (31% ± 1.2) respectively (Fig. 3A). Results showed that the transformation efficiency increased steadily in accordance with the bacterial density and reached the highest transformation efficiency at OD<sub>600</sub> 0.6, however the bacterial density was higher than that resulted in lower transformation efficiency.

#### 3.2.2. Effect of co-cultivation period on the transformation efficiency of *D. Lasianthera*

Co-cultivation is one of important steps in transformation mediated by *Agrobacterium*. After being infected by *Agrobacterium tumefaciens*, the explants are usually co-cultivated first in regeneration medium. During co-cultivation period, *Agrobacterium tumefaciens* will transfer T-DNA which bring certain gene into plants genom. In the study, we selected five different durations for co-cultivation 1–5 days. Co-cultivation period of a day produced efficiency of transformation (25.2% ± 0.7), 2 days (40% ± 2.0), 3 days (45% ± 1.2), 4 days (60% ± 1.5). The highest efficiency of transformation (65% ± 1.0) was obtained when *protocorm* and *Agrobacterium tumefaciens* had been co-cultivated for 5 days period and the lowest efficiency of transformation (25.2% ± 0.7) was obtained when *protocorm* had been co-cultivated with *Agrobacterium tumefaciens* for a day only (Fig. 3B).

#### 3.2.3. Effect of acetosyringone concentrations on the transformation efficiency of *D. Lasianthera*

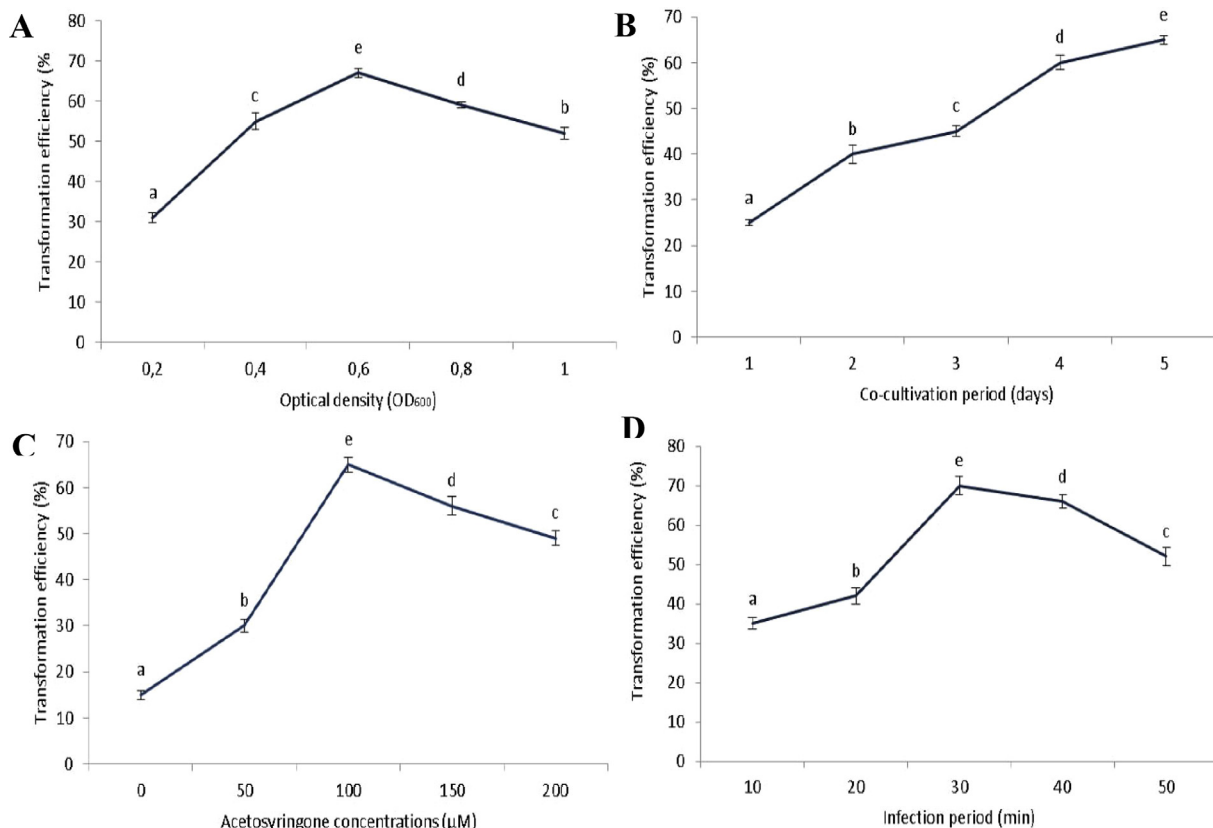
The genetic transformation mediated by *Agrobacterium* needs to transfer a single stranded T-DNA from *Agrobacterium* to plant cell, including vir genes induction. Acetosyringone has a significant role in increasing vir genes induction which causes activation of vir genes to transfer the T-DNA into plant genom. To investigate the effect of acetosyringone on transformation efficiency, different concentrations of acetosyringone (0, 50, 100, 150, and 200 μM) in the co-cultivation medium were tested. The results (Fig. 3C) showed that the lowest transformation efficiency (15% ± 1.0) was obtained for explants without acetosyringone treatment. Transformation efficiency increased as the increase of acetosyringone concentration up to 100 μM and maximum transformation efficiency (65% ± 1.5) was observed at 100 μM.

#### 3.2.4. Effect of infection period on the transformation efficiency of *D. Lasianthera*

The infection period of *Agrobacterium* determined the success of transformation. In the study, we selected five different infection period (10, 20, 30, 40, and 50 min). The efficiency of transformation was 35% ± 1.4, 42% ± 2.1, 70% ± 2.3, 66% ± 1.8, and 52% ± 2.2 when the infection period was 10, 20, 30, 40, and 50 min, respectively. The result of this study (Fig. 3D) showed that an infection period of 30 min produced the highest efficiency of transformation (70% ± 2.3) compared to infection period of 10 min (35% ± 1.4), 20 min (42% ± 2.1), 40 min (66% ± 1.8), and 50 min (52% ± 2.2).

### 3.3. Detection of putative transgenic using PCR analysis

Lane 3–7 contained the PCR products from shoots transformed with *A. tumefaciens* strain LBA4404 carrying *Knat1* gene. A single



**Fig. 3.** Effects of various factors affecting transformation efficiency of *D. lasianthera*. A bacterial density, B co-cultivation period, C acetosyringone concentrations, D infection period.

band of 1200 bp was observed on lanes 3–7 containing PCR products from putative transformer. The presence of *Knat1* gene in putative transformer confirmed the successful transformation event and supported the observation that the transformed protocorm survived on the selection media containing kanamycin.

#### 3.4. Regeneration of putative transgenic plants

Putative transgenic shoot having a length more than 1 cm was sub-cultured on RIM medium and root appeared after 3 weeks of culture (Fig. 5E).

### 4. Discussion

#### 4.1. Determination of kanamycin sensitivity

Sensitivity test of target tissue toward antibiotic is an important step in transformation [17–19]. Non-transformer tissue sensitivity test toward antibiotic was done first on regeneration medium which contained various antibiotic concentration. The lowest antibiotic concentration that can inhibit or turn out the target tissue can be used as an agent to select transformer tissue. Based on this study (Fig. 2), we found that kanamycin 75 mg/L was the lowest concentration which was able to kill a non-transgenic protocorm and the best concentration for transformer selection. For our further studies, we used 75 mg/L kanamycin as the selection agent. Several authors have been successful in using kanamycin 75 mg/L as a selection agent of transformation on different plants that are *Withania somnifera* [12] and transgenic *Urochloa brizantha* [20]. However, Mu et al. and Aggarwal et al. reported that kanamycin with lower concentration 15 mg/L and 50 mg/L were suitable for use in *Cerasus humilis* and *Eucalyptus tereticornis* [21–22].

#### 4.2. Optimization of factors influencing transformation efficiency

Several factors such as bacterial density, co-cultivation period, acetosyringone concentrations, and infection period that influenced efficiency of transformation are illustrated in [Fig. 3].

The bacterial density in suspension may influence efficiency of transformation [23–25]. The transformation efficiency described in (Fig. 3A) was obtained from 5 treatments, each treatment showed significant result (DMRT,  $p < 0.05$ ). The lowest transformation efficiency ( $31\% \pm 1.2$ ) was obtained from treatment  $OD_{600nm}$

0.2. This could be the result of inadequate number of *Agrobacterium tumefaciens* cells to infect and transfer T-DNA to protocorm cells. This claim was supported by An et al. stating that  $OD_{600nm}$  0.2 was too low, hence there was a few of *A. tumefaciens* that would transfer the T-DNAs to target cells and cause low transformation efficiency [26]. Protocorm treated with *Agrobacterium tumefaciens* on  $OD_{600nm}$  0.2, 0.4, 0.6 produced transformation efficiency that steadily increased, following after, the transformation efficiency decreased on  $OD_{600nm}$  0.8 and 1.0. The highest efficiency of transformation ( $67\% \pm 1.2$ ) was reached on treatment with  $OD_{600nm}$  0.6. The same result has been reported by Subramaniam et al. Shrestha et al. and Zhang et al. that bacterial density of  $OD_{600nm}$  at 0.6 yielded the highest transformation efficiency on *Dendrobium Savin white*, *Vanda*, and *Cattleya* [27–29]. Therefore,  $OD_{600nm}$  0.6 was used for transformation of *D. lasianthera*.

Co-cultivation period was started from 1 day until 7 days [30–33]. The results of observation (Fig. 3B) depicted that the longer co-cultivation, the more efficient the transformation and it clearly showed significant results among 5 different treatments (DMRT,  $p < 0.05$ ). Shorter co-cultivation period (1–3 days) generated low efficiency of transformation, it could be stated that co-cultivation period of 1–3 days lacked of time for *A. tumefaciens* to transfer T-DNA into protocorm cells of *D. lasianthera*. The co-cultivated protocorm for 5 days produced the highest efficiency of transformation ( $65\% \pm 1.0$ ), but it also resulted in a high bacterial overgrowth and necrosis of explants. Therefore, a 4-day co-cultivated period was used for transformation system for *D. lasianthera*. Similar results were reported by Gnasekaran et al. that 4-day co-cultivation period was suitable for use in transformation of *Vanda kasem's* [9]. However, co-cultivation for longer time (15 days) was used in *Helianthus annuus* [34].

The success of transformation mediated by *A. tumefaciens* was interfered by the presence of acetosyringone in co-cultivation medium. Various acetosyringone concentrations 0–400  $\mu$ M had been used for genetic transformation [35–37]. The result (Fig. 3C) illustrated that there were significant differences among five treatments (DMRT,  $p < 0.05$ ). The highest efficiency of transformation ( $65\% \pm 1.5$ ) was reached on co-cultivation medium given 100  $\mu$ M of acetosyringone. Higher concentrations of acetosyringone resulted in decreasing of transformation efficiency. The same result have been reported by Kartikeyan et al. Duan et al. Hosein et al. and Afolabi et al. It was reported that acetosyringone concentration of 100  $\mu$ M yielded the highest transformation efficiency on Rice,

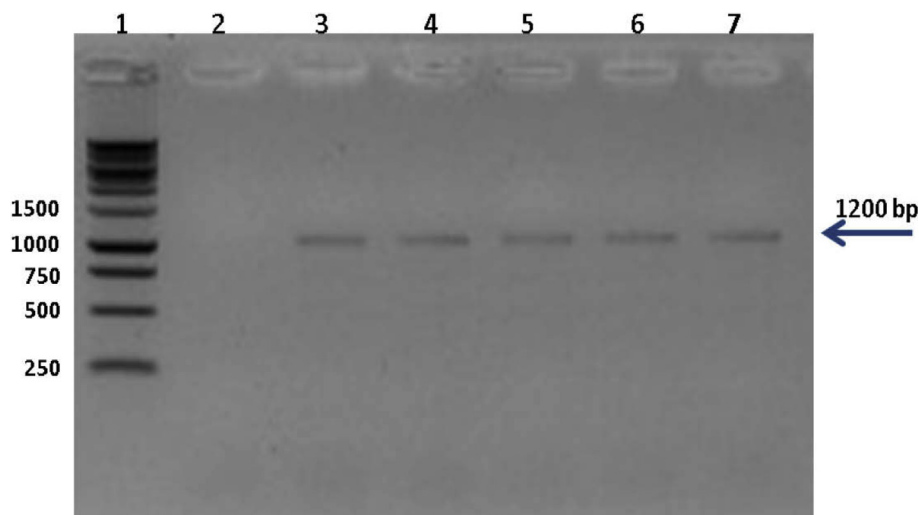
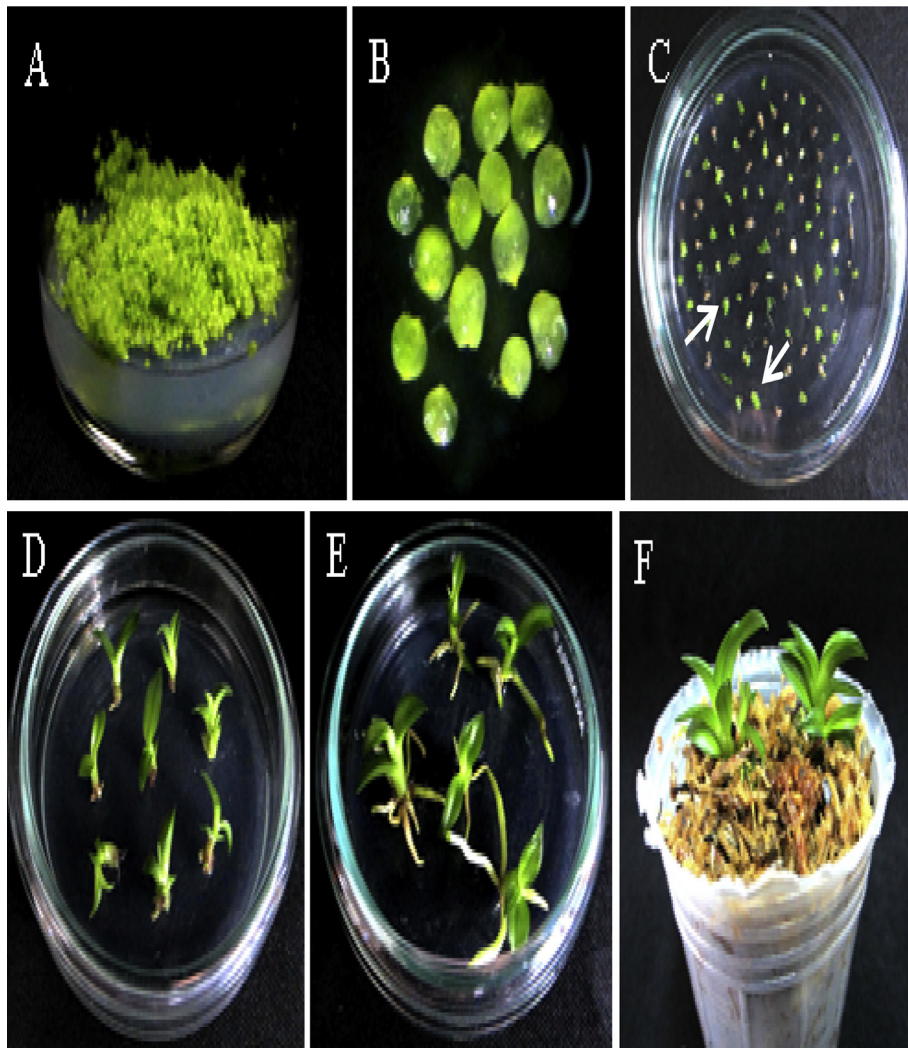


Fig. 4. Polymerase chain reaction analysis of transgenic *D. lasianthera* using *Knat1* primers. 1 = marker, 2 = wild type, 3–7 = transgenic plants (arrow = *Knat1* amplified size 1200 bp).





**Fig. 5.** Seed germination and regeneration of protocorm *Dendrobium lasianthera*. (A) Seed germination on VW medium + 3 g/L peptone + 30 g/L sucrose. (B) Protocorms were used as target of transformation. (C) Transgenic protocorms were cultured on SM medium (Arrow indicated of transformed protocorms), (D) Well developed shoots from protocorms were cultured on SIM medium. (E) Rooted plantlet were cultured on RIM medium, (F) Transgenic plant grew on mixture of coconut fiber and sphagnum moss.

*Nicotiana*, *Anthurium*, and *Cotton* [38–41]. Therefore, acetosyringone concentration of 100  $\mu\text{M}$  was further used in the transformation of *D. lasianthera*. Our result is contrastive to Rashid et al. and Suratman et al. which added acetosyringone in higher concentration (150  $\mu\text{M}$  and 200  $\mu\text{M}$ ) and produced the increase of transformation efficiency on *Wheat* and *Citrus vulgaris* [35,37]. The differences between the results might due to genotype variation.

Any results of transformations from previous research indicated that infection period varied from few minutes to few hours, 5 min on *Artemisia carvifolia* [5]; 30 min on *Oncidium* Gower Ramsey, *Crambe abyssinica*, and *Dendrobium chrysotoxum* Lindl [42–44]; 40 min on *Cordyline fruticosa* [45]; an hour on *Helianthus tuberosus* [46]; 4 h on *Erycina pusilla* [10]. The results of observation (Fig. 3D) indicated that infection period 30 min was optimum for transforming *D. lasianthera* protocorm. Since there were significant differences (DMRT,  $p < 0.05$ ) among treatments, 30 min was chosen as the infection period in order to get the highest efficiency of transformation. Men et al. stated that 30 min of infection period on *Dendrobium nobile* generated a higher efficiency of transformation (18%) rather than infection period of 45 min and 60 min [47]. The results of the study also indicated that infection period of 10 min and 20 min shorter generated lower efficiency of transformation that were  $35\% \pm 1.4$  and  $42\% \pm 2.1$ . An infection period of

40 min and 50 min longer also yielded reduction of transformation efficiency  $66\% \pm 1.8$  and  $52\% \pm 2.2$ , and overgrowth of *Agrobacterium* on the surface of protocorm led to necrosis.

#### 4.3. Molecular analysis of the putative transformer

The results of PCR analysis (Fig. 4) revealed that 1200 bp *Knat1* transgene had been successfully amplified from putative transformer kanamycin resistant. Non-transformer plant (wild) was used as control, and it showed no band amplified from them in PCR analysis. This proved that protocorm *D. lasianthera* had been successfully transformed mediated by *Agrobacterium tumefaciens* strain LBA4404 to express *Knat1* gene.

## 5. Conclusion

In conclusion, a simple and optimized *Agrobacterium*-mediated genetic transformation protocol has been established for *Dendrobium lasianthera* using protocorms explants and has been demonstrated molecularly from the integration of transgene into the genome of orchids. Transgenic plantlets were successfully regenerated. Thus, this protocol has the potential to be applied for transformation of other medicinal orchids.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgement

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# 14

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