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Development of essential skills from a research project in biotechnology: coelomic fluid of earthworm induces adventitious roots in tobacco plants

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***Abstract*- The goal of the study was to develop necessary skills for B. Tech students through an undergraduate project in biotechnology. Understanding of biotechnology involves keen observation of results and asking systematic and logical questions to find out the causes of key observation. This paper introduces teaching and attainment of essential skills during execution of their final year project. The project was focused on identifying the significance of coelomic fluid (CF) of earthworm in plant tissue culture medium by using the model plant tobacco. Project outcomes are: understanding the purpose of an experiment, conducting experiments in basic and applied areas of biotechnology, interpretation of results and communicate clearly during viva-voce examination and documenting the results in a scientific way. Essential skills viz: reading and understanding the literature, how to design an experiment with proper controls, logical explanation of results and communication skills were evaluated every month by rubrics designed by investigators. Assessment was further done by feedback system and surveys from the students. The project outcomes were matched with essential skills and their attainment was measured. Based on the evaluation it is concluded that this research project-based learning helped students to develop essential skills such as designing of experiments, logical explanation of results, communication and documentation of their observations with a conclusion in a scientific way.**

Keywords: **coelomic fluid; earthworm; project-based skills; rubrics; tobacco**

JEET Category: **Research**

I. INTRODUCTION

Project-based learning is one of the important research methodologies used by the research students such as B. Tech students. Project-based learning allows the students to gain necessary knowledge, skills, and techniques in a given time period to work on complex problems in the field of biotechnology. Expected major outcomes of the project are: understanding the purpose of an experiment, designing an experiment with proper controls, interpretation of results and present their observations and conclusions in a scientific way. Project-based learning helps the students to solve complex research problems which require solution at earliest.

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We have used project-based learning to describe the effect of CF from earthworm in plant tissue culture medium by using the model tobacco plant stem explants. Students were initially asked to read literature review on CF of earthworms to identify the research gaps in relation to plant tissue culture. Students realized that the knowledge on the effect of CF in model plant tobacco is not available.

Hence, it was decided to find out the significance of CF in plant tissue culture medium with the model plant tobacco for their final year project. As the research project demands to develop essential skills such as designing experiment, communication and documenting the results in scientific way, they were monitored periodically and measured and mapped with B. Tech program outcomes as described by National Board of Accreditation (NBA) in India.

II. LITERATURE REVIEW

Project-based skill development has been used in biotechnology for studying the advanced topics such as genetic engineering, cellular respiration, biodiversity, ecosystem, and evolution **(**C. Cruz & C. Rivera, 2022). Project-based learning has been used in the field of biotechnology to create metacognitive awareness among the students (Husamah, 2015a). During the project-based learning, skills such as self-regulated, critical and creative thinking have been reported (Husamah, 2015b). Quantitative and qualitative research methods were used by the students in broad fields of biology-based projects (Özarslan& Çetin, 2018). Blended learning is used by certain educational institutions along with project-based learning (Yustina et al., 2020). Research project-based teaching system has been developed to understand molecular biology for under-graduate students (Zhang, 2008). There have been instances of connecting biology and organic chemistry laboratory courses through collaborative research projects (Boltax et al., 2015). In an earlier report, project-based approach was used to study complex topics such as cell biology at molecular level (Wright & Boggs, 2002). There has been a report of development of the laboratory project enhanced the students understanding in cell and molecular biology (Aronson & Silveira, 2009). Project-based molecular biology study on protein structure and function has been implemented in introductory biology laboratory course (Treacy et al., 2011).

Similarly, students in our institution have done a

project-based learning in an undergraduate (B. Tech) program. Many medicinal plants and horticulture crops are vegetative propagated with stem cuttings and it depends on initiation and development of AR (Geiss et al., 2009 & Fajinmi et al., 2023). Vermiculture related products such as vermicompost, vermin tea and vermiwash had been shown to promote the growth of the crop plants in the field (Kawicha et al., 2020). Microorganisms such as bacteria or fungi isolated from these products have been shown to produce plant hormones like auxin or cytokinin (Matteoli et al., 2018). Earthworms are beneficial in identifying mitogenic and antimitotic compounds from plants (Rajamanikkam et al 2019, Rajesh et al., 2021, 2023). Moreover, CF of earthworm had been shown to increase the growth of plant cells and animal cells (Nadana Raja Vadivu et al., 2019, Nadana et al., 2020 & Subbiahanadar Chelladurai et al., 2021). The CF of earthworm had been used in plant tissue culture medium along with vermiwash or vermicompost extract and was shown to promote the callus or vegetative growth of plants (Kashyap et al., 2019). However, the effect of CF alone with plant tissue culture medium on tobacco plants is not yet studied.

III. METHODOLOGY

A. Essential skills and assessment

Ten students, three different groups of students, are involved in this project and their skills were measured periodically every month by group discussion and viva-voce examination. Engineering concepts based on National Board of Accreditation (NBA) were implemented in this learning. Each course outcome of this project is representing particular essential skill. All experiments were done at least 3 times with 3 replicates and they were led by a different student each time. Tools such as rubric score sheets (Table I), plagiarism analysis, surveys with questions related to the project (Table II) were used to evaluate students' skills. Research guide made sure that all experiments were designed properly for the research project and the results were interpreted logically. Students' feedback was received after monthly viva-voce examination and after each group discussions in order to understand their levels of skill development.

TABLE I
RUBRICS FOR ASSESSMENT DURING MONTHLY VIVA-VOCE EXAMINATION

Skills/Marks rating	Below average (2)	Average (3)	Good (4)	Very Good (5)
Reading literature and able to design new experiment	Not able to understand the literature completely	Able to understand the literature but do not know what to do	Able to understand the literature and knows what to do	Able to design new experiment to bridge the gap in literature
Able to do experiment and not doing logical explanation of results	Can do the experiment but able to explain the results partially	Can perform experiment and able to explain the results well	Can interpret the results very well with citing notes from literature	Can interpret the results logically and conclude correctly
Communication skills and tools used	Not able to convey clearly	Conveyed with grammatical errors	Oral presentation was done clearly & fluently	Used proper tools to convey better
Documentation of results in scientific way	Documentation was done with errors	Report was free from errors but poor alignment	Documentation was done in scientific way	Results were cited and documented well

Group discussion and viva-voce review were taken as direct assessment (80%) and course exit survey and 4 different feedback questionnaires were taken as indirect assessment (20%).

B. Plant materials and growth hormones

Tobacco plants (*Nicotiana tabacum* L. cv. Wisconsin 38) were obtained by students from Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Tamil Nadu, India (Sunitha et al., 2012). All the chemicals were bought from HiMedia laboratories, to grow the plants in MS medium with nutrients (PT088), 3% of sucrose (PCT0607) and 0.8% agar agar (GRM666). Earthworm species, *Eudrilus eugeniae* was purchased and cultured by students in our laboratory as described earlier (Nadana Raja Vadivu et al., 2019).

TABLE II
INDIRECT ASSESSMENT- SAMPLE QUESTIONNAIRE

1	Why do we need to study literature review for a particular research project? A) To spend time B) To support research publication C) To help research supervisors D) To find the research gap on a particular research project
2	How do you make logical explanation of a result from an experiment? A) From previous experiments' results B) By comparing with proper controls C) By checking with aim of the experiment. D) None of the above
3	Choose the wrong sentence A) All plant cells are totipotent nature B) All animal cells are also totipotent C) Explant subculture is possible in plant tissue culture D) Root and shoot induction from explants depend on plant hormones
4	What is required for good oral communication skills? A) English grammar knowledge is essential B) Speaking in small sentences is good way of beginning C) Keep practicing in speaking in English is helpful D) All of the above
5	How team-work helps in projects? A) Works can be divided to everyone and get it done fast B) It triggers communication between group members C) One can ask doubts within the group and get answered D) All of the above

The following techniques were used by students to prepare and sterilize growth hormones. 10 mg of indole-3-acetic acid (PCT083) or indole-3-butyric acid (PCT0804) was dissolved in 200 µl of 1N NaOH (MB095) solution and made-up to 10 ml with sterile distilled water. It was filter sterilized under aseptic condition using Acrodisc® 0.2 µm syringe filter (PN 4612) after making 1mg/ml stock solution. The students used IAA and IBA in the concentrations of 2mg/L in all experiments. Equipment

used for experiments were calibrated as per manufacturer's instructions, before the use.

C. CF preparation and dilution

Extrusion of CF by cold shock method was done as described earlier (Rajesh et al., 2019). To remove debris from CF students, perform a centrifuge at 1000 rpm for 1 minute. Three different dilutions of CF were prepared by adding 1ml of CF with 9 ml, 19 ml or 49 ml of MS medium to make 10-, 20- or 50-times dilutions, respectively. The volume of CF was adjusted based on the volume of MS medium used and autoclaved subsequently before the use.

D. Protein estimation

Extruded cold CF was autoclaved at 121°C for 20 minutes, and subsequently, 1 ml was used to estimate the protein concentration. Students used Bradford's method for the protein quantification, a kit purchased from HiMedia (Bradford, 1976). Commercially available bovine serum albumin (BSA) protein was purchased and used to prepare the standard graph. The R square value was 0.997, and the concentration of protein was calculated using the formula Y=0.001X + 0.233. Students repeated all the experiments at least three times, with three replicates for each sample. The standard deviation was calculated to make sure that the difference observed between each sample was statistically significant.

E. Salkowski's test

Subsequent to the removal of debris from the CF, it was transferred to fresh Eppendorf tubes and concentrated by using a speed-vacuum centrifuge at 4°C. The final approximately 20 µl sample from five individual Eppendorf tube samples was combined and made-up to 1 ml with sterile distilled water and used in Salkowski's test. The students used Salkowski's test, which estimates the concentration of indole-ring containing compounds by the colorimetric method (Gordon & Weber, 1951). Salkowski's reagent was prepared by students using 0.5M ferric chloride, 35% perchloric acid, and distilled water at a ratio of 1: 24.5: 24.5, respectively. Synthetic plant hormone IAA with different concentrations (1, 2, 3, 4, and 5µg/mL) was used to prepare the standard graph. And the R^2 value was 0.995, and the concentration of indole compound in the 5 ml CF was calculated using the following formula: Y = 0.037X - 0.004.

F. GC-MS analysis

Students concentrated the autoclaved CF using a speed vacuum at 4ºC until completely evaporating the water. Dried powder (or) CF crude powder was dissolved in 99% ethanol. GC-MS (Agilent Technologies- model 7820A series) fitted with a detector VL-MSD (model-5977E) was used to identify the volatile compounds. Sample was automatically injected into the column (DB-5) with the injector temperature at 270ºC.

IV. RESULTS AND DISCUSSION

A. Designing an experiment

CF of the earthworm had been shown to increase the rate of germination and vegetative growth in *V. radiata* seedlings (Nadana Raja Vadivu et al., 2019). However, the effect of CF in plant tissue culture medium using the model plant tobacco is not known yet. Group discussion among the students and project guide led to the decision of using tobacco explants, the stem with the shoot apical meristem. To identify suitable concentration of CF, experiment was designed with three different dilutions of CF such as 10, 20, or 50 times with MS medium. The students observed CF diluted 10 times induced adventitious roots (AR) from the stem explants after 7 days of post-subculturing (DPS) as shown in Fig. 1. Development of AR was observed by students after 11 DPS when CF was diluted 20 times and after 18 DPS when CF was diluted 50 times (data not shown). In control explants, where MS medium was used without CF, AR was initiated at the beginning of the fourth week of subculturing. These results clearly demonstrated that the roots were induced better when the CF was diluted 10 times. It is consistent with a previous report where similar diluted CF was found to induce the germination and growth of legume seedlings (Nadana Raja Vadivu et al., 2019).

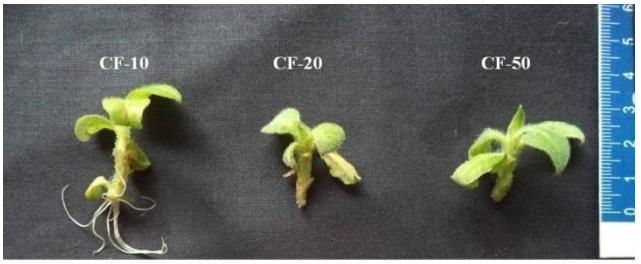

Fig. 1. CF induces adventitious roots when diluted at 10 times with MS medium. CF was diluted at 10, 20 or 50 times with MS medium and autoclaved. Stem explants with shoot apical meristem were used. Photograph was taken after 7 DPS. A ruler with measurements of centimeter is shown at the right side.

B. Logical analysis skill

Asking logical question from the results obtained is an important skill. From literature it was clear that plant hormones play a major role in development of roots and shoots development. Hence during the group discussion, the question was raised as, whether the CF has any hormone like activity. It helped them to design next experiment to find out whether CF has better root inducting ability than commercially available plant hormones. Synthetic plant root inducing hormones IAA and IBA were used to compare the effect of CF on the induction of AR. Stem explants having shoot apical meristem were chosen as explants. Surprisingly, AR was generated from stem explants treated with CF (Fig. 2) after 7 DPS. Plant hormones IAA or IBA treated explants induced the roots after 13 DPS (data not shown).Group discussion, monthly viva-voce examination and indirect assessment helped to identify where students had problem and research guides helped them to clear their doubts and solved the problem.

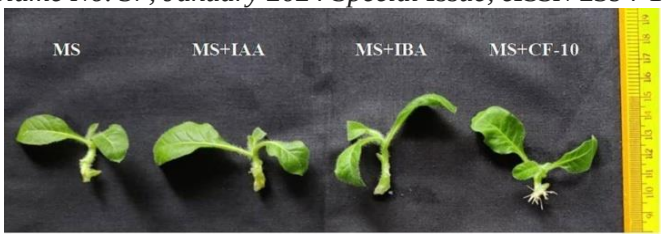

Fig. 2. Comparison of CF with synthetic plant root inducing hormones, IAA and IBA. Explants grown at plant tissue culture medium (MS), or with IAA (MS+IAA), or with IBA (MS+IBA) or with 10 times diluted CF are shown. Photograph was taken after 7 DPS. A ruler with measurements of centimeter is shown at the right side.

Systematic analysis clearly demonstrated that 10 times diluted CF in MS medium induced rapid AR induction in tobacco stem explants either with or without shoot apical meristem. CF acts as a natural organic alternate root inducing compound compared to synthetic root inducing plant hormones. Putative compounds present in the CF had already been analysed by GC-MS and LC-MS and shown to have plant growth promoting substances such as heneicosane and cadaverine (Nadana Raja Vadivu et al., 2019&Nadana et al., 2020). The plant tissue culture medium (MS) is enriched with macro and micronutrients and hence the nutrients in the CF might not be cause for development of AR. The presence of proteins or enzymes present in the CF might have not supported the root development as CF was autoclaved along with MS medium. Proteins and enzymes would be denatured during the process of autoclaving and may not be biologically active, however, it might become into amino acids and acts as supplement for the growth and development of tobacco stem explants. Estimation of proteins in the autoclaved CF revealed that the concentration of proteins was at 250µg/ml. The protein BSA (Bovine serum albumin) was added at 300µg/ml with MS medium and it was found out that it did not induce AR in the stem explants. Hence, it ruled out the possible role of nutrients or proteins from CF in induction of AR. However, it is very clear that autoclaved CF possesses root promoting compounds.

C. Communication skill

During the group discussion it was found out that vermicompost-based products promoted growth and development, and microorganisms present in the vermicompost were suggested for the production of plant hormone-like compounds. Bacterial and fungal isolates identified from the vermicompost-based products have been shown to produce plant hormone-like compounds (Kawicha et al., 2020 & Matteoli et al., 2018). The cytokinin class of plant hormones had already been identified from vermicompost tea by the LC-MS method (Zhang et al., 2013) and hypothesized as a product of microbes in the soil. During the viva-voce examination it was asked whether the CF has any auxin-like compounds. Oral communication with research guide helped to decide the execution of Salkowski's test to detect the presence of any indole-based organic compounds in CF. Initial experiments performed by students with CF on diluted or undiluted samples did not identify any such compound, as there was no pink colour in the biochemical test. It was assumed that hormones may be present in trace amounts in the CF and support the growth of plants. In order to concentrate the unknown compound, the CF was vacuum centrifuged at 4°C. Students used five ml of autoclaved CF, which was concentrated and examined with Salkowski's test. Now they found the presence of an indole-type compound at a concentration of 1.4 µg (in 5 ml of CF), as shown in Fig. 3. For 50 ml of MS medium, students used 100 µg IAA or IBA to induce AR, whereas 5 ml of CF containing 1.4 µg of an indole-based compound induced better AR. Oral communication with students revealed the previous findings in literature that CF has a mixture of various compounds (Nadana Raja Vadivu et al., 2019 & Nadana et al., 2020). Further oral communication led to the conclusion that the role of break-down products of CF after the autoclaving process is not known. Moreover, other compounds in the CF, besides indole-ring containing compound, maybe beneficial in AR generation from tobacco stem explants.

Further group discussion with supervisor and information gathered from literature made them to perform GC-MS to identify the unknown indole compound (Fig. 4). Based on the mass value, students predicted the compound 1H-Indole, 5-methyl-2-phenyl (IMP). This indole-related IMP compound has never been reported before from CF of earthworm or vermicompost based products. However, this IMP compound was reported from two bacterial strains found in stored rice grains and has antifungal activity (Mannaa& Kim, 2018).

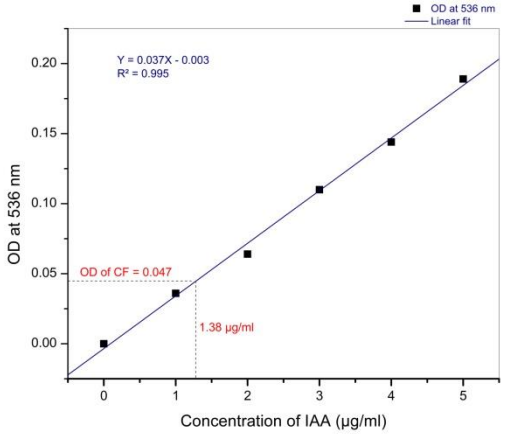

Fig.3. Estimation of indole-related compound by Salkowski's test. Standard graph was drawn with different known concentrations of IAA. Concentration of an indole compound in CF (µg/ml) was calculated using the equation Y = 0.037X - 0.003 with an R^2 value of 0.995, where Y is absorbance and X is concentration.

Recently, an IMP compound has been found in bee hives and acts as a repellent to a particular beetle species (Dekebo& Jung, 2020). Previous studies on IMP suggest that this compound appears to be beneficial to earthworms, as they need it to survive in the soil environment against different insects and microorganisms. Indole derivatives are known to have many biological and pharmacological activities, including a role in plant growth and development (Singh & Singh, 2017). However, the molecular mechanism of root initiation or development in tobacco stem explants induced by the IMP compound is yet to be discovered. This rapid and robust AR development could be due to the combinational effect of many compounds present in the CF, as reported earlier by LC-MS and GC-MS

studies (Nadana Raja Vadivu et al., 2019 & Nadana et al., 2020). In this report, the ethanol fraction of autoclaved CF identified IMP as one of the major compounds in the fluid. Induction of AR in tobacco stem explants was not observed when the CF was sterilized with 0.2 µm filter and used in MS medium (data not shown). It suggests that coelomocytes or microorganisms present in the CF might have released this IMP compound during the process of autoclaving and supported the induction of AR.

D. Assessment of skills

To determine the importance of the indole ring in inducing AR in laboratory conditions, the students used the amino acid tryptophan at different concentrations along with MS medium. We discovered that the AR were nicely induced from the stem explants (without shoot apical meristem) when tryptophan was used at a concentration of 4 µg/ml along with MS medium, compared to other concentrations such as 1, 2, 3, or 5 µg/ml. Students observed that induction of roots occurred after 11 DPS in tryptophan-containing medium, whereas roots were induced after 13 DPS by the hormone IAA (data not shown).

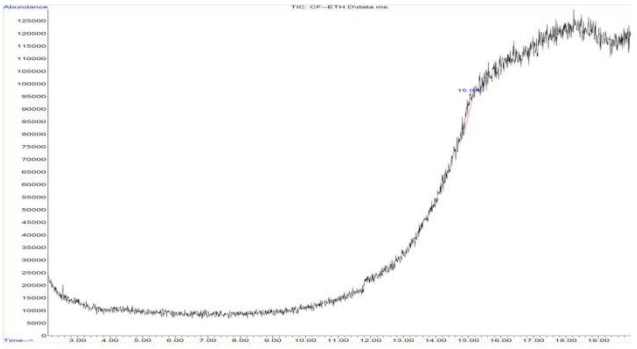

Fig.4. GC-MS chromatogram of autoclaved CF. Concentrated CF was suspended in ethanol and subjected to chromatography.

The amino acid leucine, which lacks an indole ring, did not induce AR within 13 DPS. Our results clearly demonstrated that there is a strong relationship between AR development and indole-ring containing compounds. Amino acid L-tryptophan had already been shown to induce AR in an ornamental plant (Bergonci et al., 2023). The biosynthesis of the plant hormone IAA, either tryptophan-dependent or independent, had been reported earlier to play a role in the vegetative growth of tobacco plants (Sitbon et al., 2000). In *Arabidopsis thaliana* plants, serotonin, a tryptophan-derived compound, has been shown to induce AR at low concentrations (Pelagio-Flores et al., 2011). Serotonin is also shown to be necessary for the social ability of the earthworm (Gopi Daisy et al., 2016).

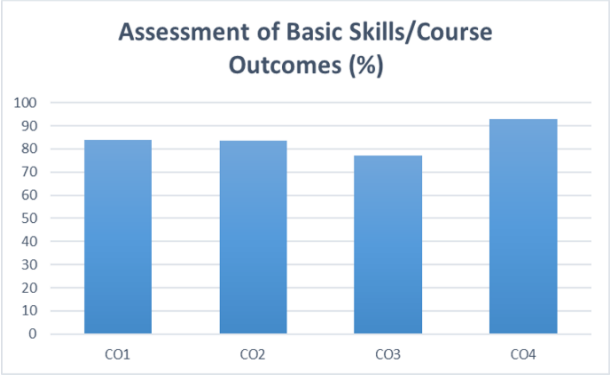

Fig.5. Assessment report of essential skills. Reading relevant literature and finding the research gap (CO1), designing experiment and execution (CO2), communication skills (CO3) and documentation of results with conclusion (CO4) are calculated and represented in percentage.

Previous studies indicate that there is a strong correlation between indole-based compounds (IAA, IBA, serotonin, and tryptophan) and the development of AR in plants.

AR is initiated by the activation of meristematic cells present near the pericycle layer in the vascular bundles of stem cuttings (Rotblat et al., 2002). By an unknown mechanism, compounds in the CF of the earthworm initiate active mitotic cell division to develop root primordia from the stem explants. Group discussions and viva-voce examinations were taken as direct assessment (80%) and course exit-survey and unanimous surveys with questionnaire were considered as indirect assessment (20%) and their skills were evaluated. Evaluation with rubrics helped the students to assess their level of strength in every skill. Students' skill in designing and execution of experiment improved over the period of 3 months due to group discussions. Their skills on interpretation of results and documentation were periodically verified and suggestions were given for improvement. Except communication skills all other skills were estimated at more than 80% (Fig. 5) at the end of the project.

V. CONCLUSION

Students' essential skills such as finding research gaps by reading literature, designing and performing an experiment, interpretation of results and communicating with research guide and documenting them in scientific way were improved during their final year's research project. Group discussions with research guide and reviews increased their communication skills and logical thinking on designing next experiment. Discussion on synthetic plant hormones and their role on induction of roots in plants made students to ask whether CF has any indole-ring containing compounds. At the end of the project, they could discover one indole-ring containing compound in the CF of earthworm. The new putative indole-derivative, either directly or with combination of other compounds present in the CF, supported rapid initiation of AR and the growth of tobacco explants. Assessment methods indicated that research ecosystem helped them to improve essential skills.

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