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PROJECT-BASED LEARNING: PURIFICATION AND CHARACTERIZATION OF 2,4-D, LINKING CHEMISTRY AND PLANT TISSUE CULTURE

APRENDIZAJE BASADO EN PROYECTOS: PURIFICACIÓN Y CARACTERIZACIÓN DEL 2,4-D, VINCULANDO LA QUÍMICA Y EL CULTIVO DE TEJIDOS VEGETALES

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Resumen

El Aprendizaje Basado en Proyectos (ABP) ofrece un marco centrado en el estudiante que integra el conocimiento teórico con la experimentación práctica, promoviendo la autonomía, la colaboración y el pensamiento crítico. Este enfoque es especialmente significativo en las ciencias experimentales, como la química, donde la práctica de laboratorio es esencial. En este trabajo, los estudiantes de bioquímica desarrollaron un proyecto ABP centrado en la obtención, caracterización y evaluación biológica del ácido 2,4-diclorofenoxiacético (2,4-D), una auxina sintética ampliamente utilizada. Los estudiantes extrajeron 2,4-D a partir de una formulación comercial de sal de dimetilamina mediante precipitación ácida, filtración y recristalización. Los métodos de caracterización, incluidos el análisis de punto de fusión y la espectroscopía IR, confirmaron la identidad y la pureza del compuesto. Aunque el rendimiento alcanzó solo el 57,66%, demostró la viabilidad de obtener material utilizable de calidad reactivo a partir de fuentes de bajo costo. Para evaluar la actividad biológica, el 2,4-D purificado se aplicó a cultivos *in vitro* de *Lupinus mutabilis*, una leguminosa Andina con notable valor nutricional y agronómico. La inducción de callo varió según la concentración de la hormona, no hubo respuesta en el control, mientras que la concentración más alta (6 mg L^{-1}) produjo un callo compacto y de rápido crecimiento, lo que confirmó la integridad funcional del compuesto purificado. Estos resultados coinciden con los patrones conocidos de dediferenciación dependiente de auxinas en leguminosas. En general, este trabajo de ABP fortaleció las competencias prácticas de los estudiantes, su capacidad de resolución de problemas y su razonamiento científico.

Palabras clave: ABP; 2,4-D; inducción de callo; Educación en Bioquímica; *Lupinus mutabilis*

Abstract

Project-Based Learning (PjBL) offers a student centered framework that integrates theoretical knowledge with hands on experimentation, fostering autonomy, collaboration, and critical thinking. This approach is particularly meaningful in experimental sciences such as chemistry, where laboratory practice is essential. In this work, biochemistry students developed a PjBL project focused on the isolation, characterization, and biological evaluation of 2,4-dichlorophenoxyacetic acid (2,4-D), a widely used synthetic auxin. Students extracted 2,4-D from a commercial dimethylamine salt formulation through acid precipitation, filtration, and recrystallization. Characterization methods, including melting-point analysis and IR spectroscopy, confirmed the identity and purity of the compound. Although the yield reached only 57.66%, it demonstrated the feasibility of obtaining usable reagent-grade material from low cost sources. To assess biological activity, purified 2,4-D was applied to *in vitro* cultures of *Lupinus mutabilis*, an Andean legume with significant nutritional and agronomic value. Callus induction varied with hormone concentration, no response occurred in the control, while the highest concentration (6 mg L^{-1}) produced compact, rapidly growing callus, confirming the functional integrity of the purified compound. These results align with known auxin-dependent patterns of dedifferentiation in legumes. Overall, this PjBL work strengthened students' practical competencies, problem solving abilities, and scientific reasoning.

Keywords: PjBL; 2,4-D; callus induction; Biochemistry education; *Lupinus mutabilis*

1. Introduction

Project-Based Learning (PjBL) is an inquiry-based, student-centered educational approach in which the students engage in real-world projects to integrate theory with practice. It promotes autonomy, collaboration, and critical thinking also improving cognitive, affective, and behavioral skills essential for professional competence (Aley et al., 2024). In this context, implementing PjBL in disciplines such as chemistry becomes particularly relevant, as chemistry (according to Hofstein & Hugerat, 2022) cannot be effectively taught without the laboratory experience especially at the university level. In our country, Bermeo et al. (2018) identified deficiencies in infrastructure (including limited availability of equipment and reagents) as a significant factor contributing to student repetition. Moreover, the budget for higher education was US$ 1.319 billion in 2024, almost US$ 3 million less than in 2023 (SENESCYT, 2024). Frequent budget cuts in higher education limit the ability to provide students with a practical education, consistent with the development of science.

Within this context, a PjBL for Biochemistry students at the Central University of Ecuador, Faculty of Chemical Sciences was aimed at proposing a methodology for the isolation of a chemical compound of interest. Here we describe the isolation, characterization and application in plant tissue of 2,4-dichlorophenoxyacetic acid (2,4-D), a common, cheap and accessible herbicide in order to address the lack of this reagent due to budget limitations. Interestingly the theoretical basis, methodology and laboratory procedures were all proposed and developed by the students under the guidance of the Biochemistry laboratory staff. The students applied qualitative, quantitative and instrumental techniques that they have learned throughout their coursework and obtained 2,4-D from a commercial herbicide source.

Auxins are a group of plant natural hormones that regulate plant growth and development. The chemical compound 2,4-D is one of the most common synthetic auxins used as herbicide. After application, it is absorbed through the roots and leaves within 4 to 6 hours and then distributed via phloem. 2,4-D affects plant metabolism by interfering with food transport, overstimulating the growth of young cells and rejuvenating old cells, leading to abnormal growth patterns and death in some plants, specifically it eliminates broadleaf plants (Kennepohl et al., 2010). Its chemical structure is characterized by a benzene ring substituted at positions 2 and 4 with chlorine atoms and at position 1 with a phenoxy group (Figure 1a). The molecular formula of 2,4-D is $C_8H_6Cl_2O_3$, it´s molecular weight is 221.038 g/mol, forms crystals in benzene, the melting point is 140 °C, it´s boiling point is 160 °C (0.4 mm Hg), insoluble in water, soluble in ethanol, and slightly soluble in benzene and DMSO (Haynes et al., 2016). The basic form of 2,4-D is the acid, but it is usually formulated commercially as an inorganic salt, amine (Figure 1b) or ester (Kennepohl et al., 2010).

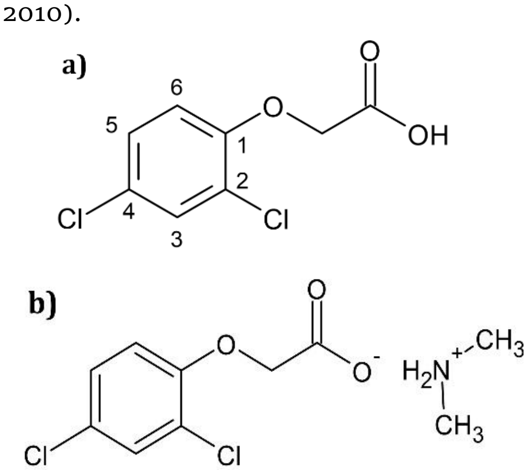

Figure 1. Chemical structure of 2,4-D a) acid form showing two chlorine atoms and phenoxy group b) 2,4-D dimethylamine salt.

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Plant tissue culture is understood as the methodologies that use aseptic culture of cells, tissues or organs, under defined *in vitro* conditions (physical and chemical). Among these 2,4-D is used for cell division, root initiation and morphogenesis suppression, the latter is known as callus induction. For this purpose, the common concentration for 2,4-D is a stock solution of 10 mg/100 mL (Thorpe, 2021).

The following PjBL proposed the application of the purified 2,4-D as a synthetic auxin to evaluate its ability to induce callogenesis in *in vitro* cultures of *Lupinus. mutabilis* (chocho). This species is a legume native to the Andes with high nutritional value due to its protein content, which can reach 35% to 45% on a dry basis, in addition to its lipid profile rich in unsaturated fatty acids (Pszczółkowski et al., 2015). Its ability to fix atmospheric nitrogen and thrive in low-fertility soils makes it a relevant species for sustainable agricultural systems (Villacrés et al., 2006). (Thorpe, 2021).

2. Methodology

2.1 Reagent source and assays

A commercial solution of 2,4-D dimethylamine salt with a common concentration of 60 g/100 mL was used. From this, 5 mL (arbitrary volume) where taken and placed into a beaker, then a solution of HCl 1M was added in order to fulfill the reaction described in Figure 2.

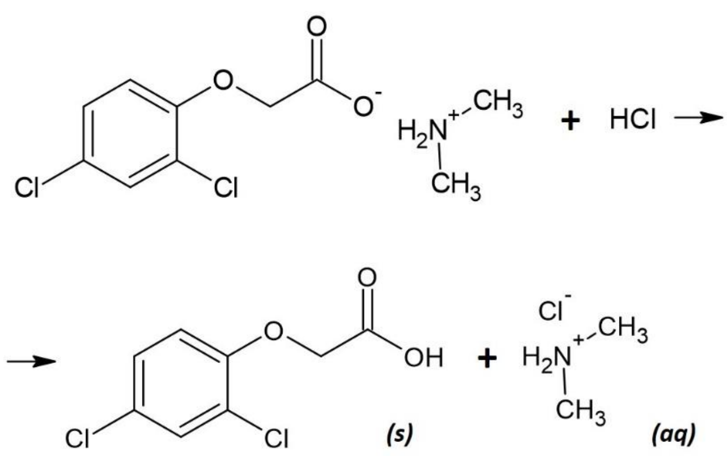

Figure 2. Reaction of 2,4-D precipitation from 2,4-D dimethylamine salt

The solid formed was allowed to stand for a few minutes and then was transferred to a Büchner funnel and filtered under vacuum using a Ningbo Maisi Diaphragm Vacuum Pump TC-100. The solid was subjected to a re-crystallization process in 96% ethanol at room temperature on a Petri dish for 24 hours. The re-crystallized product was dried. Finally, the product obtained was characterized by melting point measurement and IR spectroscopy by Attenuated Total Reflectance (ATR) method. The melting point was determined with a Büchi melting point apparatus and the IR was performed with a FT-IR Jasco 4600 spectrophotometer. The yield of the isolation was calculated.

2.2 Plant material and tissue culture assay

The explants *of L. mutabilis* were obtained from seedlings previously germinated in sterile substrate and subsequently transferred to the laboratory for *in vitro* establishment. Surface disinfection was performed by immersion in 96% ethanol and subsequent treatment with 10% sodium hypochlorite, a procedure widely recommended for leguminous species due to its effectiveness in reducing microbial contamination without affecting plant tissue viability. Under aseptic conditions in a laminar flow cabinet Faithful CJ-1DII, the meristem apical segments were placed in half-strength Murashige and Skoog (MS) medium supplemented with sucrose and gelled with agar. Depending on the treatment, the media were enriched with 2,4-D at concentrations of 0, 2, 4, and 6 mg L^{-1}, following previously described criteria for the induction of callogenesis in species of the genus Lupinus (Salcedo-Morales et al., 2024). Subsequently, the cultures were maintained at 25 ± 2 °C with a photoperiod of 16 h light/8 h darkness, and the morphogenetic response of the explants was evaluated weekly for two weeks. This protocol was previously standardized in our Laboratory and was based from the available literature (Gessesse & Feyissa, 2021; Varma & Jain, 2021).

3. Results

3.1 Purification and charaterization of 2,4-D

Based on the concentration of 2,4-D stated on the label of the commercial bottle, the theoretical amount of 2,4-D contained in 5 mL of initial solution (arbitrarily set) was calculated as:

$$w_{theoretical} = 5mL \cdot \frac{60\ g\ 2{,}4\text{–}D}{100mL}$$

$$3.0\ g\ 2{,}4\text{–}D$$

From this value, the volume (mL) of HCl required to reach the pH between 1.5 to 2 was calculated as:

$$V_{HCl} = 3.0\ g\ 2{,}4\text{–}D \cdot 1mol \frac{H^+}{221{,}038\ g\ 2{,}4\text{–}D} \cdot 1000ml \frac{H^+}{1\ mol\ H^+}$$

$V_{HCl} = 13.57\ mL\ H^+$

$$(for\ 5mL\ considered\ in\ this\ assay)$$

The addition of 13.57 ensured to reach a pH needed to protonate organic acids. The acid caused the immediate precipitation of a white solid inside the beaker, the solid was then filtrated by vacuum (Figure 3a). The solid was then re-dissolved in 7.5 mL of 96% ethanol (the minimum volume required for crystal dissolution) and finally, after re-crystallization, a white amorphous crystal solid was obtained (Figure 3b).

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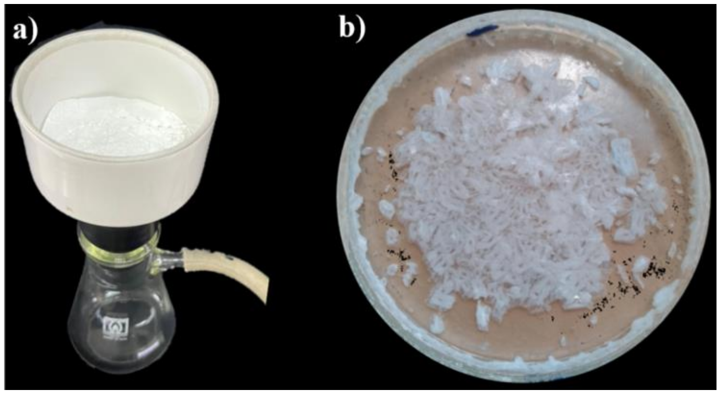

Figure 3. Isolation of 2,4-D a) Büchner funnel with precipitate b) recrystallized 2,4-D

The melting point was measured between 135.7 °C and 143.5 °C, with an average value of **140.5 °C**. The IR spectrum obtained (Figure 4a) displayed several characteristic absorption bands corresponding to aromatic groups, a carbonyl stretch, and a distinct fingerprint region similar to the IR spectrum described elsewhere (Figure 4b).

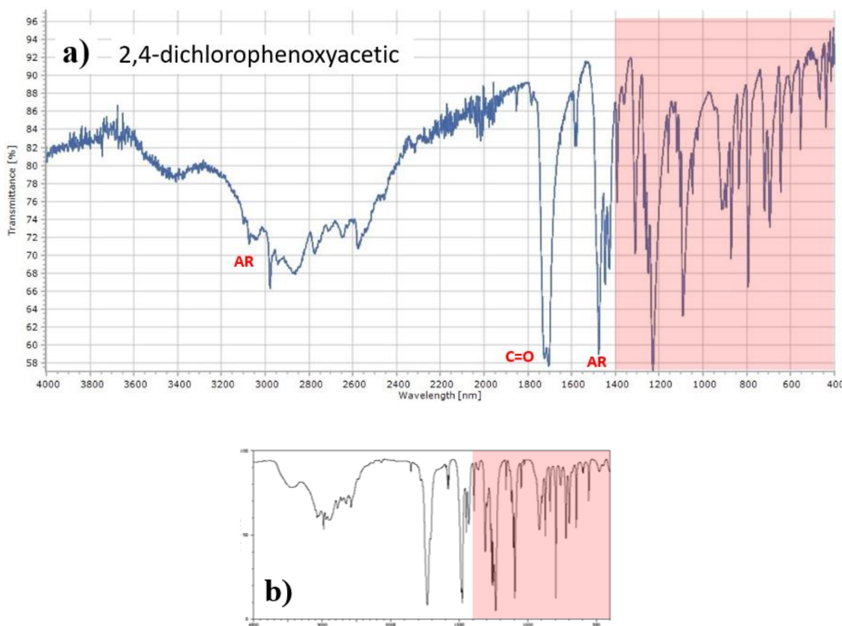

Figure 4. 2,4-D IR spectrum a) IR obtained in this study (main peaks in red) b) reference IR spectrum (Chemical Book, 2024). Both spectra show the fingerprint region (pink area).

Finally, the weight of 2.4-D obtained in the present study was 1.7298 g, which represented an isolation yield of 57.66 in comparison with de theoretical weight of 3.0 g.

3.2 Callus formation in *L. mutabilis* explants

Variations on the response of the explants were seen depending on the concentration of 2,4-D. In the absence of hormone (0 mg L^{-1}), no callus formation was observed (Figure 5a). At 2 mg L^{-1} a moderate positive response was recorded (Figure 5b) while the concentration of 4 mg L^{-1}

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increased the formation of dedifferentiated tissue (Figure 5c). The greatest response was obtained with 6 mg L^{-1} of 2,4-D, where the explants developed compact callus and accelerated growth during the evaluation period (Figure 5d).

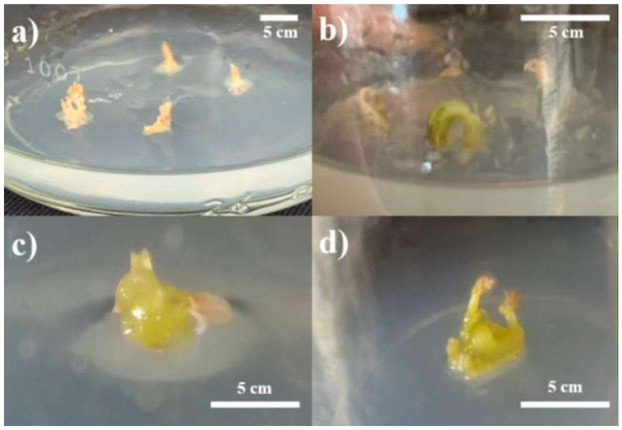

Figure 5. Callus formation a) Explants at 0 mg L^{-1} of 2,4-D, no callus were observed b) Explants 2 mg L^{-1} of 2,4-D, slight response c) Explant at 4 mg L^{-1} of 2,4-D, higher response, possibly contaminated with bacteria d) Explant at 6 mg L^{-1} of 2,4-D, highest response was observed, growing structures similar to somatic embryo were present.

4. Discussion.

The implementation of a PjBL approach described in this work, provided students with an authentic opportunity to integrate theoretical concepts with practical laboratory practice. According to Zhang & Ma (2023) the core principles of effective project-based learning include active student engagement, the integration of theoretical knowledge with practical application, the development of higher-order thinking skills, and meaningful learning through authentic, real-world tasks, all of these aspects were successfully addressed by the students throughout the execution of this project.

From a practical perspective, although the isolation procedure successfully yielded a reagent grade 2,4-D suitable and effective for the educational purposes of this PjBL, it should not be regarded as a substitute for analytical or technical grade reagents required in formal research. As noted by Pareek (2022), educators can and should be resourceful in developing cost-effective instructional materials, but high-purity reagents remain essential for producing reliable and reproducible scientific data. Beyond these educational considerations, the chemical properties and behavior of the reagent supports that the procedure was well developed. The observed precipitation upon HCl addition agrees with the expected acid–base behavior: dimethylamine salts are soluble in aqueous media, but protonation with a strong acid releases the weak acid (2,4-D), forming the insoluble free acid and dimethylammonium chloride as a byproduct. This mechanism aligns with similar reactions reported by Arsenijević (2008).

The average melting point (140.5 °C) is consistent with the value reported by Haynes et al. (2016), confirming the correct identification of the compound. The similarity between the IR spectrum obtained in this study and the reference spectrum reported by Chemical Book (2024) further supports the successful isolation of 2,4-D. The characteristic peaks associated with aromatic C–H stretching, carbonyl (C=O) vibrations, and the fingerprint region confirm the structural integrity of the compound.

Although the yield (57.66%) was moderate, it demonstrates that a substantial amount of 2,4-D can be recovered from its commercial amine salt formulation through a simple acid–base extraction and recrystallization process. Possible losses may be due to incomplete precipitation, solubility losses during washing, or handling errors.

Callus formation in *L. mutabilis* increased proportionally with the concentration of 2,4-D, reaching its maximum at 6 mg L^{-1}. This behavior is consistent with what has been reported for other Lupinus species, where high concentrations of synthetic auxins promote cell dedifferentiation and the proliferation of unorganized tissues (Salcedo-Morales et al. 2024). In legumes, 2,4-D acts as a key regulator to activate cell division and expansion pathways, especially in juvenile explants, which explains the marked response observed in your study (Gessesse & Feyissa, 2021).

The absence of response in the treatment without growth regulators and, additionally, the acquisition of a whitish coloration, indicative of a lack of active cell division and possible mild oxidative stress, also coincides with the literature. Callogenesis in Lupinus is highly dependent on exogenous auxins due to the low endogenous production of these compounds in somatic tissues (Asghar, Ghori & Hyat 2023). On the other hand, the positive response observed at intermediate concentrations (2 and 4 mg L^{-1}) indicates that *L. mutabilis* maintains a pattern like that described in other species of the genus, where moderate doses of 2,4-D initiate callus formation but without reaching the maximum level of proliferation.

Finally, the high effectiveness of the treatment with 6 mg L^{-1} confirms that purified 2,4-D maintained its biological activity after the extraction and purification process, thus fulfilling its function as a synthetic auxin within the *in vitro* culture. This validation is especially important given the objective of the work, which is to demonstrate the viability of the compound obtained from commercial herbicide.

5. Conclusions

Given that PjBL supports the meaningful integration of theory and practice within inherently experimental disciplines, the development of this PjBL-based essay is fully justified as a suitable opportunity to apply this pedagogical approach in a context where it is particularly relevant.

We isolated 2,4-D from a commercial source as an exercise for Biochemistry students, we are confident that this study has contributed to the students' ability to develop skills to adapt to constraints such as limited resources, thus gaining valuable experience in problem solving and innovative thinking in a chemical research context.

The two characterization tests of the solid obtained lead us to conclude that 2,4-D was successfully isolated from commercial herbicide. We finally demonstrated the biological activity of the purified 2,4-D in *L. mutabilis* cultures and gave students direct evidence that their chemical product was functional. The observed increase of callus formation at 6 mg L^{-1} of 2,4-D provided not only an applied context for plant tissue culture but also a concrete indicator that their extraction process was successful. In order to statistically validate the results, further experiments should be done.

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