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Curriculum

Isolation and Characterization of *Agrobacterium* Strains from Soil: A Laboratory Capstone Experience †

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In this investigation, the students' goal was to isolate and characterize *Agrobacterium* strains from soil. Following selection and enrichment on 1A-t medium, putative *Agrobacterium* isolates were characterized by Gram stain reaction and biochemical tests. Isolates were further evaluated using polymerase chain reaction (PCR) with different primer sets designed to amplify specific regions of bacterial deoxyribonucleic acid (DNA). Primer sets included AGRH to identify isolates that were members of the *Rhizobiaceae*, BIOVAR1 primers to identify members of *Agrobacterium* biovar group I, and a third set, VIRG, to determine presence of *virG* (only present in pathogenic *Agrobacterium* strains). During the investigation, students applied previously learned techniques including serial dilution, use of selective/differential media, staining protocols, biochemical analysis, molecular analysis via PCR, and electrophoresis. Students also gained practical experience using photo documentation to record data for an eventual mock journal publication of the capstone laboratory experience. Pre- and post-evaluation of class content knowledge related to the techniques, protocols, and learning objectives of these laboratories revealed significant learning gains in the content areas of *Agrobacterium*–plant interactions ($p \leq 0.001$) and molecular biology ($p \leq 0.01$). The capstone journal assignment served as the assessment tool to evaluate mastery and application of laboratory technique, the ability to accurately collect and evaluate data, and critical thinking skills associated with experimental troubleshooting and extrapolation. Analysis of journal reports following the capstone experience showed significant improvement in assignment scores ($p \leq 0.0001$) and attainment of capstone experience learning outcomes.

INTRODUCTION

Agrobacterium is a Gram-negative, rod-shaped soil bacterium of the *Rhizobiaceae,* and it is the causative agent of crown gall disease in plants. This disease results from the unique ability of the pathogen to interact with plant cells and transfer part of its own deoxyribonucleic acid (DNA) to the host cell. Due to its distinctive mode of infection, *Agrobacterium* is a natural genetic engineer of plant cells. To initiate infection, bacteria invade plant tissue through wounds in the plant. Once inside plant tissue, *Agrobacterium* binds to the cell wall and transfers small fragments of DNA from the tumor inducing plasmid (Ti) into the nucleus of the host cell. The transferred DNA (T-DNA) contains genes that cause plant cells to overproduce hormones such as auxin and cytokinin (3). Hormone overproduction in these transformed cells results in cell proliferation and the production of a gall. Although crown gall disease is not a problem for most crop plants, it is a nuisance for certain plants, notably grape, *Euonymus*, and rose. Not all isolates of *Agrobacterium* have the ability to induce gall formation. Some strains are unable to recognize and colonize wounded plant cells, while other strains contain modified hormone biosynthesis genes, which either elicit no response or cause only root formation. Other avirulent strains may lack the Ti plasmid containing the virulence genes (including *virG*) that ultimately facilitate the transfer of T-DNA to plant cells (3, 10).

Students in a biology majors microbiology course do not often receive practice isolating and identifying organisms from rich resources such as soil, and given the abundance of microbes in the rhizosphere, it can be difficult to isolate and characterize various species due to sheer numbers of microbial possibilities. This laboratory sequence introduced students to soil microbes with the application of previously learned classical techniques and expanded those approaches to include molecular identification of a microbe at the family, biovar, and virulence level. Polymerase chain reaction

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(PCR) primer set design was based upon a tiered strategy. One set of primers (AGRH) identified all soil isolates of the large *Agrobacterium*/*Rhizobium* group. The BIOVAR1 primer set further identified the subset of biovar 1 isolates that are typically found in association with groups of plants including rose and *Euonymus*. The VIRG primer set identified a small subset of biovar 1 isolates that are pathogenic and induce gall formation. In designing the capstone experience, we hypothesized that if students engaged in an investigative laboratory sequence related to *Agrobacterium* isolation and characterization, they would master related techniques and content as well as improve higher-order thinking skills (5) as demonstrated through data recording and production of a journal-quality laboratory report. All strategies for isolation and identification used in this investigation were outgrowths of a successful research laboratory project (1).

Intended audience

This project was designed for an undergraduate upper-division biology majors course. With substitution or addition of an infectivity assay or additional molecular analysis, the set of laboratories would also be appropriate for an undergraduate course in either plant pathology or plant molecular biology.

Prerequisite student knowledge

Students will have been introduced to all basic microbiology laboratory techniques (culture transfer/aseptic technique, staining, microscopy, isolation, and biochemical identification) and biosafety level 2 (BSL2) laboratory safety protocols (11) in the first nine weeks of the semester. Also, it is advantageous (although not required) for students to have been introduced to electrophoresis principles and techniques in prerequisite courses such as cell biology.

Learning time

A minimum of six laboratory sessions, 1.5 hours each, are required to complete all components of isolation and identification. Depending upon the time of year isolation is attempted, it may take three to five days to see putative *Agrobacterium* colonies appear on 1A medium amended with tellurite (1A-t). The earliest growth seen will appear in 48 hours. Thus, depending upon timing of colony appearance, time allocated to the investigation may need to be extended.

Learning objectives

During this investigation and following completion of the laboratory sequence, students will be able to:

1. Demonstrate content knowledge in the following areas: laboratory techniques, molecular biology, *Agrobacterium*–plant interactions, and electrophoresis
2. Implement learned laboratory techniques (serial dilution, selective/differential media use and interpretation, biochemical testing, staining, and microscopy)
3. Explain the roles of *Agrobacterium* as a pathogen and natural genetic engineer
4. Describe how PCR can be used for molecular characterization of isolates
5. Analyze and evaluate PCR results relative to *Agrobacterium* identification using three different primer sets
6. Demonstrate proficiency at recording and processing scientific data resulting in production of a journal-quality laboratory report

PROCEDURE

Materials

Materials and equipment are listed for a class of 12 to 18 students working in pairs. Additional details regarding materials (including media recipes) are found in Appendices 1 and 2.

Laboratories 1 and 2

- Student-collected soil samples in clean plastic bags (one sample per group)
- Sterile water, 2-mL microfuge tubes, micropipetters (200 μL and 1000 μL) and tips for serial dilutions
- Sterile, disposable plastic loops (500 per class), or wire loops (two per group)
- 1A-t medium (eight plates per group)
- Yeast extract peptone (YEP) agar (eight plates per group)
- 28°C incubator (one per class)

Laboratories 3 and 4

- Gram stain reagents and microscope slides (box of 100 per class)
- Oxidase tests (one per group)
- Sulfide-indole-motility (SIM) agar, Nitrate broth (three of each per group)
- Benedict's reagent (100 mL per class)
- 3-ketolactose agar (one plate per group)
- YEP agar (four plates per group)

Laboratories 5 and 6

- Wild-type virulent *Agrobacterium tumefaciens* culture
- 2% Triton X-100/1% sodium azide solution (200 μL per group)
- PCR reagent kits (30 reactions per class)
- Primer sets for PCR (30 reactions per class)
- Heating block, ice bath, high-speed centrifuge, thermocycler (one of each per class)

- Agarose gels and gel electrophoresis equipment (three to four gels and electrophoresis apparatuses per class)
- 100 bp DNA ladder (20 μL per class)
- Ultraviolet (UV) trans-illuminator/photo-documentation system for gel viewing (one per class)

For all laboratory sessions

- Laboratory conditions to support BSL2 including restricted laboratory access (including signage), hand-washing stations, personal protective equipment (including gloves, eye protection, and laboratory coats), appropriate sharp and biohazardous waste disposal (autoclave), available biosafety cabinets, student safety training module/manual.

Student instructions

Student instructions are available in Appendix 1. Although most major details are provided, several parameters may be modified by the student. Because this set of laboratories is meant to be investigative and reflect an approach followed in the research laboratory, groups should be encouraged to make changes to the protocol as needed depending upon their samples, preliminary results, and literature searches.

Faculty instructions

A short summary of the student laboratory sessions appears below. In addition, Appendix 2 contains advice and cautions for instructors regarding each laboratory session as well as potential modifications and pitfalls. Laboratory preparation information and media recipes are also found in Appendix 2.

Laboratory 1. Students arrive at the laboratory session with collected soil. Following creation of a soil slurry, students carry out serial dilutions and plate aliquots on 1A-t medium. Plates are incubated at 28°C for two to three days or until colonies are visible.

Laboratory 2. Students select a minimum of three dark, round, glistening colonies and sub-culture (streak for isolation) each colony onto 1A-t and YEP media and incubate for two days. Students should enrich and select for pure cultures that can be used in the next laboratory to biochemically characterize isolates.

Laboratory 3. Biochemical tests inoculated include 3-ketolactose plates, SIM agar deeps, and nitrate broth. Students also perform catalase and oxidase tests and Gram stain each isolate with results recorded in this session. Cultures should be re-streaked on YEP medium for the next laboratory.

Laboratory 4. Students add reagents to biochemical tests and record results. Putative *Agrobacterium* colonies are re-streaked on YEP agar for the next laboratory session.

Laboratory 5. By laboratory 5 each pair of students should have three biochemically characterized isolates. Isolates are used for colony DNA preparation, with the DNA serving as the PCR template. A student group with three isolates prepares nine PCR reactions (three reactions/primer sets per isolate) and runs the reactions in the thermocycler. Completed reactions should be held at 4°C until the next laboratory session. A *virG* positive *Agrobacterium* isolate should be processed parallel to the students' samples to serve as the positive control for all three primer sets.

Laboratory 6. PCR products are electrophoresed and results recorded (photography or digital imaging). All plates, biochemical tests, reagents, and gels should be discarded after laboratory completion.

Suggestions for determining student learning

Pre- and post-laboratory content tests were administered to students addressing the following four major subject areas (subscales) covered in the capstone experience: laboratory techniques, *Agrobacterium*–plant interactions, molecular biology, and electrophoresis. The test consisted of 33 questions distributed among the aforementioned four categories (Appendix 4). Pre- and post-capstone experience scores were compared and evaluated using paired sample *t*-tests and Wilcoxon signed-rank tests to examine the impact of the capstone experience on mastery of covered content (learning outcome 1). To determine if individual students exhibited improved higher-order thinking (learning outcomes 2 to 6), the formal laboratory report was evaluated using a custom rubric (Appendix 3) and those scores compared with scores from a previous laboratory report, written during week 7 of the semester.

Sample data

When growing on 1A-t medium, members of the *Rhizobiaceae* (Fig. 1) produced black, glistening, smooth, raised colonies with round margins within five days of dilution plating (2, 9). Typically 10^{-1} and 10^{-2} dilutions of soil extracts allowed for growth of isolated colonies with minimal overgrowth by other organisms. Candidate *Agrobacterium* isolates presented as Gram-negative bacilli that were oxidase positive, producing a dark purple color on oxidase dry slides immediately (Fig. 1). Most environmental isolates were nitrate and motility positive. Putative biovar 1 strains produced a yellow ring around the colony in the 3-ketolactose assay while 3-ketolactose negative isolates were non-reactive (Fig. 2). PCR gels included the 100 bp ladder as a size standard (Fig. 3, lane 6) as well as student and control reactions (Fig. 3). A student sample

in lane 7 with AGRH primers yielded an amplicon of 739 bp, while student samples using BIOVAR1 primers (lanes 2, 11) produced a product of approximately 473 bp. The student sample in lane 10 demonstrated several bands.

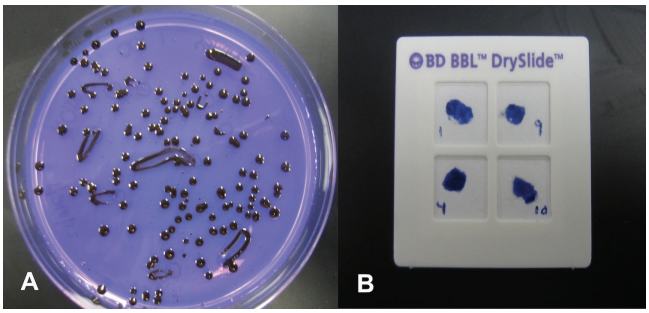

FIGURE 1. (A) Soil samples plated on 1A-t medium. Colonies are putative *Agrobacterium* isolates. (B) Positive oxidase tests.

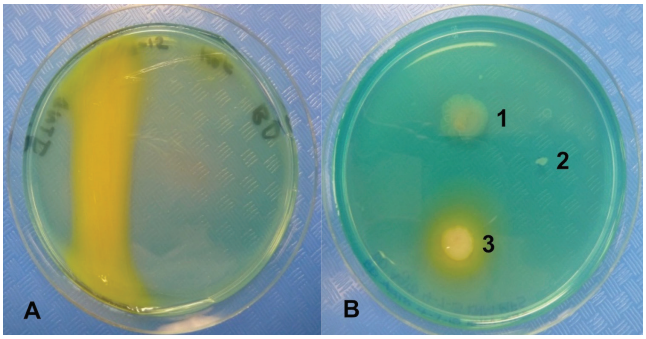

FIGURE 2. Test results for 3-ketolactose. (A) Positive control, *A. tumefaciens* EHA105. (B) 1, 2: negative isolates; 3: positive isolate.

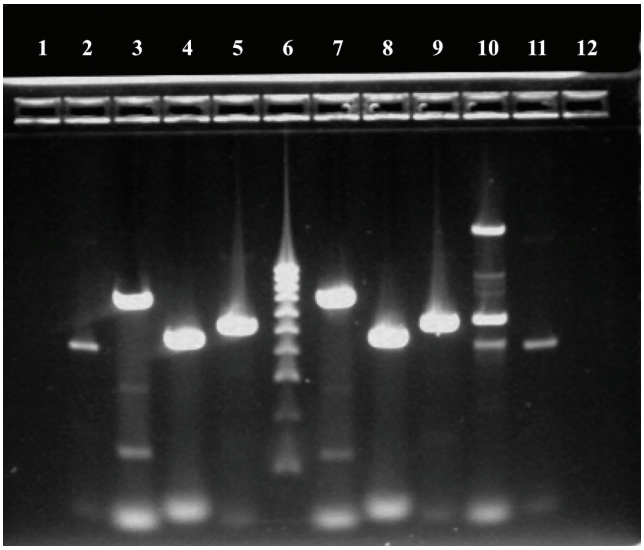

FIGURE 3. Gel electrophoresis of PCR products. Lane 6 = 100 bp ladder. Lanes 3, 4, 5, 8, 9 primer controls (3 = AGRH; 4, 8 = BIOVAR1; 5, 9 = VIRG). Student amplification products are in lanes 2 (BIOVAR1), 7 (AGRH), and 11 (BIOVAR1). The student sample in lane 10 contains amplicons from all three primer sets. Lanes 1 and 12 are empty. PCR = polymerase chain reaction.

It was later determined that the student group loaded all three PCR amplification products into a single lane. Their interpretation of results suggested isolation of a biovar 1, *virG*-positive *Agrobacterium,* although additional bands of unknown significance also appeared. The biovar 1, pathogenic strain *A. tumefaciens* EHA105 (4), served as the control strain and yielded amplicons with all three primers (lanes 3, 4, 5, 8, 9).

Safety issues

Because 1A-t medium (2, 9) contains several selective components (including cycloheximide which is toxic to humans), the majority of colonies that grew on 1A-t plates represented isolates of *Rhizobiaceae,* which are typically maintained under BSL1 conditions (ATCC, www.ATCC.org). However; various hardy species of *Pseudomonas* as well as yeast and fungi can grow on 1A-t medium (2), particularly after prolonged incubation. Thus, because of the initial unknown identity of colonies, all isolates were manipulated using BSL2 safety protocols and precautions (11). Agarose gels contained ethidium bromide and were handled with gloves and discarded as hazardous waste. Alternately, a less toxic stain for DNA such as SYBR Safe (THERMOFISHER) may be substituted for ethidium bromide. The cell-lysing solution used to isolate colony DNA contained sodium azide, a compound of known toxicity in humans and thus handled with extreme caution. If the available UV trans-illuminator is not contained but rather open, UV eye protection must be worn.

DISCUSSION

Laboratory investigation of bacterial populations of either soil or plant material provides students with unique opportunities to familiarize themselves with organisms not often emphasized in a microbiology course offered to biology majors. In this capstone experience, students were tasked with isolating members of the *Rhizobiaceae* and characterizing isolates at the family, biovar, and virulence levels using selective media and biochemical and molecular techniques. And while experimental results were unpredictable, students regularly isolated a variety of diverse *Agrobacterium* strains (Fig. 3). Laboratory skills mastered included not only experimental techniques and approaches, but also practice at recording, processing, and presenting data in a journal format. Group analysis of content mastery in molecular biology and *Agrobacterium*–plant interactions showed a significant increase in content learning as a result of this experience. The journal assignment assessment scores indicated significant use and application of higher-order thinking during completion of the assignment. A recent report by Martinez-Vaz et al. (7) also demonstrated similar impacts on student learning and critical thinking when examining unknown bacterial populations of root nodules.

Field testing

This particular laboratory investigation has served as the capstone experience in four different sections of an upper-division biology majors microbiology course offered over the past four spring semesters. During each offering, parameters of the experiments (particularly media composition and incubation parameters) were continually modified to maximize the probability of isolating *Agrobacterium* strains. After the first use of the capstone experience, an individual skill-set check was put in place to ensure student proficiency at handling microbes and utilizing aseptic technique. Skill-set checks were given during week 7 of the semester (before the capstone experience). Any student who failed to demonstrate proficiency received personal tutoring and practice outside of class time until proficiency was achieved.

Class size typically ranged from 12 to 18 students. Even student numbers allowed for experiments to be done in pairs. If the class contained an odd number of students, one group was composed of three individuals. The extensive nature of the investigation provided enough activity and data recording such that all students in the larger group of three were engaged in all steps.

Since the initial capstone offering, students have provided positive feedback through course evaluations (data not shown), particularly as related to use of previously learned techniques and a chance to apply them in experimental protocols (rather than "one and done"). Self-efficacy surveys (SENCER-SALG, http://www.sencer.net/assessment/sencersalg.cfm) revealed significant increased confidence (Table 1) in understanding the nature of science and applying learned techniques as a result of the capstone experience. In addition, two students developed a keen interest in the project and continued to search for and characterize *Agrobacterium* strains in soil samples the semester following the capstone experience.

Although most students reported enjoying the capstone experience, there may be some frustration associated with using an open-ended laboratory rather than "cookbook" exercises where outcomes are predetermined. Instructors using the capstone investigation should be aware of the following problems that may arise:

- Because 1A-t medium is selective and incubation is at 28°C, colonies can be slow to form (2 to 5 days), and growth rates may be associated with seasonal temperatures (9). Therefore, the laboratory sequence may have to be expanded to accommodate slow growth of organisms.
- Although 1A-t medium is selective and differential for *Agrobacterium*, overgrowth of various soil organisms can become problematic. Potential isolates include *Pseudomonas* sp., yeast sp., and *Actinomycetes* sp. Overgrowth is likely to occur as incubation periods are extended.
- Environmental isolates may vary in phenotype from known laboratory strains (i.e., *Agrobacterium tumefaciens* EHA105); thus it is important to emphasize the possibility of strain variability with regard to biochemical test results (particularly if students decide to examine carbohydrate fermentation reactions).
- Given the paucity of *virG*-positive isolates in soil (1, 9) many samples must be screened to find virulent strains. Thus students may not isolate any *virG* positive strains in the capstone experience.
- PCR may fail to yield any amplicons (or produce amplicons in sizes that are unexpected). Student disappointment may be addressed by talking about the nature of science and experimentation and the influence of variables. In a research laboratory, rarely are optimal results achieved the first time an experiment is run; rather, experimental parameters must be modified and hypotheses reformulated as necessary.

Evidence of student learning

There was a significant difference in the pre- and posttest scores (percent correct) for the overall assessment (33 questions) of comprehension (Table 2). A paired samples *t*-test indicated that posttest scores were significantly higher (64.9) than pretest scores (52.7). When subscale (question categories) findings were examined separately, there was a significant difference in the posttest scores for the molecular biology subscale (6.5) versus the pretest scores (4.6). There was also a significant difference in the posttest scores (7.9) and the pretest scores (6.0) for the *Agrobacterium*–plant interactions subscale. Due to the small sample size of the course section (n = 16) Wilcoxon signed-rank tests were used to compare median values of the overall assessment and the four subscale pre/posttest

TABLE 1.
Self-efficacy evaluation of three course sections (2011, 2013, 2015).

Subscale	Mean (n = 46)	Standard Deviation	Paired t-test (2-tailed) Sig. $p \leq$
Confidence in understanding the nature of science 11 questions (Max. score 44)	Pre 36.2 Post 42.4	5.0 1.9	0.01
Confidence in use and application of experimental techniques 9 questions (Max. score 36)	Pre 26.5 Post 33.5	3.7 2.4	0.01

TABLE 2.
Parametric (*t*-test) and nonparametric analysis (Wilcoxon signed-rank test) of student content evaluation: pre- and post-capstone experience.

Subscale	Mean (n = 16)	Standard Deviation	Paired *t*-test, (2 tailed) Sig. p =	Median (n = 16)	Wilcoxon signed-rank test (2-tailed) Sig. p =
Overall Score (%) 33 questions	Pre 52.7% Post 64.9%	12.5 13.5	0.001	Pre 57.5% Post 67.5%	0.004
Molecular Biology Number correct of 11 questions	Pre 4.6 Post 6.5	1.5 1.5	0.002	Pre 4.0 Post 7.0	0.006
Agrobacterium–plant Interactions Number correct of 10 questions	Pre 6.0 Post 7.9	1.8 1.5	0.001	Pre 6.0 Post 8.0	0.005
Laboratory Techniques Number correct of 9 questions	Pre 3.9 Post 4.0	1.3 1.7	0.669	Pre 4.0 Post 4.0	0.575
Electrophoresis Number correct of 3 questions	Pre 2.2 Post 2.3	0.75 0.77	0.580	Pre 2.0 Post 2.0	0.564

ranks. Like the *t*-test, the Wilcoxon signed-rank test analysis identified significant increases (p values) in posttest scores in the overall assessment as well as in the molecular biology and *Agrobacterium*–plant interactions subscales (Table 2). The content test did not identify a significant increase in knowledge of either electrophoresis details or laboratory techniques. This outcome was somewhat anticipated for the electrophoresis questions, as electrophoresis principles and practice were covered in a prerequisite course. However, the finding of no significant increase in content knowledge regarding laboratory techniques and biochemical identification was unexpected. One explanation for similar pre- and posttest scores in the laboratory techniques subscale may be that timing of the posttest was premature. The posttest was given before students completed their laboratory journal reports, during which more effective assimilation of biochemical details may have taken place. However, it should be noted that increased student confidence in understanding the nature of science and application of capstone techniques was significant (Table 1), and this confidence in the application of knowledge was demonstrated in the journal assignment. A paired samples *t*-test was used to analyze scores on the capstone journal assignment (n = 17, M = 41.4, SD = 3.6) and scores from an earlier (week 7 of the semester) laboratory report (n = 17, M = 37.1, SD = 4.7). This comparison served to evaluate achievement of learning outcomes and improvement in writing skills associated with data recording, analysis, and interpretation of laboratory results. Significant improvement in understanding, application, and creative thinking was noted ($p \leq 0.0001$), and use of the rubric indicated that all learning outcomes were achieved. The most notable finding was that 82% of the students demonstrated proficiency at recording and processing scientific data resulting in production of a journal-quality laboratory report thus achieving learning outcome 6 (listed as learning outcome 5 in the rubric found in Appendix 3).

Depending upon the institution, students may receive little training in recording data in a laboratory notebook and writing formal laboratory reports in their biology courses. This capstone activity serves as an opportunity for students to engage in and master those skills, which are crucial to success in the graduate programs often pursued by biology majors. Most importantly, the capstone journal assignment gives students a chance to process results, fit them into a context, draw conclusions, and provide suggestions for other approaches and experimental modifications. This requires utilizing higher-order thinking skills (5) such as applying, analyzing, evaluating, and creating (learning outcomes 2 to 6) in a laboratory setting.

Possible modifications

Students may choose to sample plant galls rather than soil. *Euonymus* (a common ground cover or small shrub found in wooded areas and in cultivated landscapes) is exceptionally susceptible to infection and is usually a good source of these structures (Fig. 4). Rather than creating a soil suspension, students macerate surface-washed galls in sterile water and then proceed with plating of serial dilutions on 1A-t medium (2).

A pathogenic assay can be used to identify virulent strains of *Agrobacterium* rather than using PCR with the VIRG primer set. In this approach, either *Kalanchoe*—a nursery/house plant (8)—or carrot slices (6) are inoculated with student isolates. Use of a pathogenic strain in the infectivity assay will result in lesions, roots, or galls appearing on plant tissue in four weeks.

Since *Agrobacterium* is present in soil, a reverse approach to the experiment can be taken by analyzing total soil DNA (NUCLEOSPIN SOIL) with PCR for the presence of *virG*. If soil samples yield a positive signal in PCR with VIRG primers, isolation of the organism from the specific soil sample can then be attempted. Even though isolation

FIGURE 4. *Agrobacterium*-induced gall (arrow) formed in *Euonymus*.

of soil DNA and amplification of *virG* from soil DNA can be challenging, the reverse approach has the advantage of being able to quickly screen many soil samples (with only one primer set) to increase the chances of isolating a pathogenic biovar 1 strain of *Agrobacterium*.

In courses with a molecular focus, PCR amplicons may be cut out of gels, extracted, and sequenced. Additionally, strains can be further examined using amplification of the 16S gene to compare environmental isolate sequences to known *A. tumefaciens* strains (12).

SUPPLEMENTAL MATERIALS

Appendix 1: Student instructions
Appendix 2: Instructor instructions
Appendix 3: Journal laboratory report rubric
Appendix 4: Pre- and post-laboratory test questions

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