Traditional and in vitro development of new clover (*Trifolium* spp.) plants©

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Abstract

The objectives of the study were to cultivate and breed *Trifolium repens*, grow and micropropagate various species of *Trifolium,* and develop protocols for the genetic manipulation of *T. repens* in vitro. Because white clover is a self-sterile hermaphrodite, cross-pollination is necessary to create viable seed from genetically different parents. As a result of exposing *T. repens* to 6-benzylaminopurine (BAP) in vitro, adventitious shoot formation was initiated and it was observed that a concentration of 1 mg L^{-1} BAP is optimal for adventitious shoot initiation. Other species of *Trifolium* responded similarly to that of *T. repens* while cultivated in vitro. Colchicine and Surflan® (chemical mutagens) were used successfully to produce mutations in *T. repens*. The plants exposed to these mutagens demonstrated physical mutations such as an increase in leaflets per clover and thicker petiole tissue. This research provides evidence that plant tissue culture can be used to micropropagate endangered *Trifolium* species and chemically induced mutations which resulted from this study.

INTRODUCTION

The clover plant, *Trifolium* spp., comes in all shapes, sizes, and colors and provides benefits to the surrounding flora and fauna. These benefits include the fixation of nitrogen, favorable nectar and nutrient density, and potential for growth as an ornamental plant. This study utilized plant tissue culture for the development of new clover cultivars as well as for the establishment and micropropagation of several clover species, both common and endangered, in vitro. Research into the manipulation of white clover plants in vitro is limited. Therefore, the methods that were used to establish clover plants in tissue culture were based on responses of other plants in vitro (Kyte et al., 2014).

As a nitrogen fixer, clover can convert freely available nitrogen into ammonium compounds through nodules in its roots. This unique and valuable trait is a result of a symbiotic relationship between the clover plant and *Rhizobia* bacteria present in the soil (Frame, n.d.). White clover is also rich in a number of essential nutrients such as calcium, phosphorus, magnesium, potassium, and protein (Søegaard, 1993). Furthermore, white clover has been found to improve the daily gains of cattle (Hoveland et al., 1991), to benefit surrounding plants (Parente and Frame, 1993), and to correlate with an increase in carrying capacity of a pasture for deer (Stevens et al., 1992).

In a study by Quesenberry (2002), the importance of several species of clover was evaluated throughout the United States. Quesenberry (2002) estimated that the amount of nitrogen fixed by clover in the United States for 1 year would equate to $525 million based on the price of nitrogen in the form of NH_4NO_3. Quesenberry also estimated the value of good quality red clover hay to be a $6.4 billion dollar market.

White clover is a stoloniferous plant which branches out from growth nodes along its stolon. From these nodes, roots may form on the surface which is in contact with the ground; offshoots, or runners, may branch out as a form of asexual reproduction; and/or petiole and leaves may form (Figure 1). White clover, *T. repens*, is known for having white flowers and moderately sized leaflets with a distinguished white marking on its leaflets (Frame, n.d.). Red clover, *T. pratense*, is similar in structure to white clover in that it is stoloniferous,

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however, red clover is less of a cover crop and it tends to grow significantly higher off the ground than white clover. Red clover is also known for having red colored flowers as opposed to white clover's white to pink flowers (Brickell and Zuk, 1997).

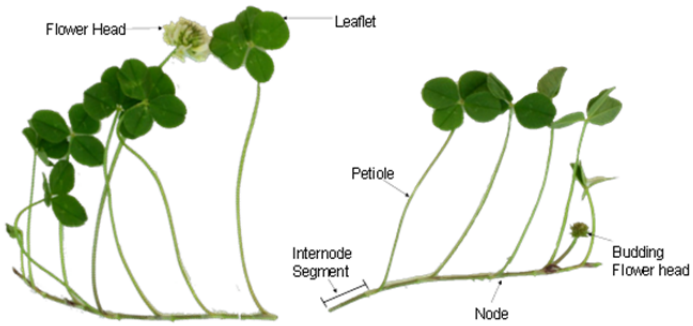

Figure 1. Structure of a white clover plant.

Driven by its benefits and potential as an ornamental plant, Lee (2007) created a new cultivar of white clover which was produced through mutagenic exposures; the new cultivar of clover was patented under the name 'Lucky Together'. By using the mutagen ethyl methanesulfonate (EMS) alongside hormones and growth regulators, Lee was able to develop a new cultivar of clover with aesthetically pleasing qualities, tolerances to Korea's environment, as well as the benefits that clover previously offered. Lee's research provides evidence to support the idea that random mutations in plants as a result of chemical exposure can yield characteristics which have aesthetic appeal as well as practical uses for society (Lee, 2007).

Bae et al. (2009) also successfully mutated and bred white clover for specific traits. One of the traits bred were multifoliate clovers. Multifoliate clovers have greater than three leaflets. The four-leaf clover is the most common naturally occuring multifoliate clover. Bae et al. (2009) used gamma radiation to mutate clover seeds while the seeds were in their developing stages. After exposure, the surviving plants were left to grow and 11.7% of the surviving population exposed to 25 Gy exhibited mutation in leaf number compared to the control which had 0% mutation in leaf number. The plants that expressed mutations in leaf number were then cultivated specifically for the multifoliate trait (Bae et al., 2009).

Polyploidy has also been induced in plants as a result of mutagen exposure. In a 2008 study, polyploidy was achieved by exposing *Rhododendron* seedlings to Surflan®, an herbicide, which contains 40.4% oryzalin, a known chemical mutagen (Jones et al., 2008). Colchicine and oryzalin are both known mutagens and have been observed to cause random mutations such as polyploidy and mixoploidy. Colchicine is a spindle fiber interrupter and is known for its use outside of the agricultural world for treating gout inflammations (Schlesinger et al., 2006). Polyploidy and mixoploidy have been recorded as a result of exposing plants to oryzalin and colchicine by Ascough et al. (2008) and Schlesinger et al. (2006). These studies further support that the chemical mutagens, oryzalin and colchicine, are capable of producing random mutations in plants.

Phenotypic mutations, i.e., physically apparent mutations, in clover plants can be observed in nature as well. One of such mutations is multifoliate clovers. Multifoliate clovers are the foundation of the legend of the "lucky" four-leaf clover.

This study approached the primary steps for development of new clover cultivars differently than previous studies. Chemical mutagens, oryzalin and colchicine were used to mutate clover plants in vitro rather than using radiation or EMS. This study also worked toward the establishment of protocol to micropropagate selections of clover, both common and endangered, in vitro. The methods that were used were derived from the existing research concerning clover plants, as well as the information which has been around on growing plants in vitro since the beginning of the 20th century (Kyte et al., 2014).

MATERIALS AND METHODS

Traditional cultivation of white clover

White clover seeds were scarified using 400 grit sandpaper to remove part of the seed coat and allow water to more readily initiate germination. The scarified clover seeds were set in petri dishes containing a paper filter and water and remained in the dishes until there were visible signs of germination (1-5 days). As clover seeds germinated, they were individually transferred into plastic greenhouse trays containing a 1:1 ratio of potting mix to sand medium. They were grown for 8-10 weeks, the point at which the plants began to flower.

When the white clover plants began to flower, those plants that expressed unique phenotypic characteristics were separated and transplanted into plastic pots. The plants were crossbred by transferring pollen from one floret to the stigma of another floret on a genetically distinct plant with a toothpick. Seeds were harvested 3-5 weeks after transferring the pollen.

Sterilization of clover seeds

Accepted standards of aseptic technique were followed while working under a laminar flow hood equipped with a HEPA filter. To be sterilized, seeds of white clover were placed into glass beakers, submerged in 95% ethanol, agitated with a swirling motion for 30 seconds, and then had the ethanol decanted. The seeds were set aflame to burn off remaining ethanol and scarify the seeds. This flaming process was repeated three times for each set of seeds to ensure sterility. Once completed, the seeds were aseptically transferred onto ¼ strength Murashige and Skoog medium in disposable petri dishes, labeled, and placed in the growth chamber to germinate.

In vitro growth regulator trials

Full strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was prepared in glass culture tubes with the following concentrations of the cytokinin 6-benzylaminopurine (BAP): 0, 0.125, 0.25, and 1.0 mg L^{-1}. The pH of the media were adjusted to a range of 5.7-5.8, agar was added 7 g L^{-1}, and culture tubes were autoclaved.

White clover plantlets were aseptically transferred into tubes of the four different BAP concentrations; there were 16 replications used per treatment. The plants were placed in a 22°C growth chamber in a randomized complete block design. After four weeks the plants had their roots and foliage removed and were subcultured onto fresh media with the same levels of BAP. After a total of 8 weeks the plantlets were removed and their shoot numbers were counted (Figure 2).

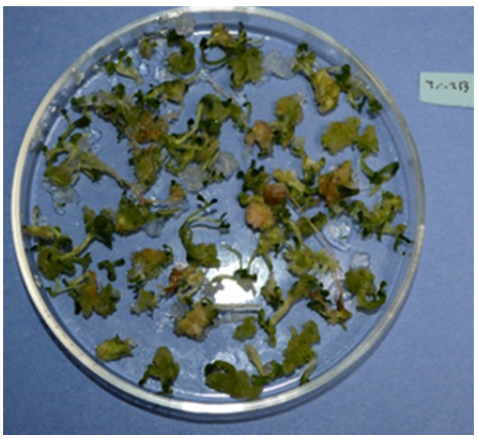

Figure 2. White clover plants produced in vitro on a Murashige and Skoog (1962) medium containing 1 mg L^{-1} 6-benzylaminopurine.

Chemical mutagen studies

1. Surflan application.

Once aseptic white clover seeds germinated, 8-day old seedlings were exposed to varying concentrations of Surflan (40% oryzalin) for three different times under the protection of a laboratory fume hood. The Surflan concentrations that the plants were subjected to were 0.1%, 0.5%, 1.0%, and a control with 0 Surflan. Plantlets in each of these four treatments were exposed to the mutagen for 30, 60, and 90 minutes. There were a total of 144 seedlings that were subjected to these treatments. Following their respective treatments, seedlings were rinsed in sterile water and remained in sterile water until subcultured into Magenta™ GA-7 culture vessels containing ¼ strength MS media; there were five seedlings per magenta. After 4 weeks, the plants were subcultured onto fresh media. After another 4 weeks had elapsed, the plantlets were subcultured again, but this time individually into test tubes containing ¼ strength MS after having their roots and foliage removed. Data on the survival of the plants exposed was recorded 8 weeks after their exposure. Approximately 5 weeks after the subculture into test tubes, the surviving plantlets were transplanted into plastic cell packs containing a perlite-peat based medium and moved to the greenhouse.

2. Colchicine application.

The other mutagen used in this study was colchicine. It was administered to the plants via the growing medium; a different manner than the Surflan. The four solutions of colchicine had concentrations of 0, 0.1, 0.5, and 1.0 g L^{-1} colchicine.

The colchicine solutions were filter-sterilized through a 0.45 µm hydrophilic cellulose acetate membrane filter. One liter of one quarter strength MS medium with 7 g L^{-1} agar was prepared and poured into 250 ml quantities prior to autoclaving. After autoclaving, each of the colchicine solutions were loaded into their respective sterile syringes connected to filter sterilizers and were pumped into the still-liquid, autoclaved media. The colchicine media were then poured into sterile petri dishes and allowed to cool and solidify under the laminar flow hood.

After the colchicine medium were cooled, plants were subcultured onto them. There were four plants placed in each petri dish. The cultured plants were then placed in the growth chamber at 21°C with 24 h lighting. After 48 h, half of the petri dishes were removed from the growth chamber and the plantlets were subcultured onto ¼ strength MS and returned to the growth chamber. Six days after the initial culturing of plantlets into the colchicine media, the plants that remained on colchicine media were subcultured onto ¼ strength media and also returned to the growth chamber. These two exposure periods provided for pulse durations of 2 and 6 days. After 5 weeks, the surviving plantlets were subcultured into culture tubes containing ¼ strength MS after having their roots and foliage removed. After 5 additional weeks, the plantlets were transplanted into cell packs containing a peat and perlite mix and moved to the greenhouse where they were acclimated. Plantlets that initially exhibited unique phenotypic characteristics were identified and rather than moved to the greenhouse, were subcultured onto fresh medium and remained in the growth room.

RESULTS AND DISCUSSION

Traditional cultivation of white clover

Scarification of white clover seeds with 400 grit sandpaper allowed for sufficient removal of the seed coat for improved germination. The scarified seeds began to swell within 24 h after imbibition of water. The cultivation of white clover under the conditions outlined in the methods sustained healthy plants through seed harvest. Hybridization procedures consistently produced 1 to 3 seeds per fertilized ovary.

The successful harvest of clover seeds was delayed due to the self-sterility of clover. The plants that were initially cross-pollinated were clones of each other, so no viable seed

was able to be harvested. It was not until genetically distinct plants were cross-pollinated that viable seed was able to be harvested. These hybrid seeds were harvested 3-5 weeks after pollination, or when the flower head had dried out entirely and the seeds were able to easily fall out of the florets. The seeds ranged in color from yellow to brown but all maintained similar size. The viability of the seeds harvested was verified by scarifying 12 of the harvested seeds and repeating the germination process. Offspring that were grown from hybrid seed and produced from parent plants with multifoliate clovers also produced multifoliate clovers. Some of the offspring raised adopted the distinguished white "v" mark variegation present in the male parent while the remaining offspring expressed the trait with less opaqueness.

In vitro growth regulator trials

The results from these experiments demonstrate that the concentration of 1.0 mg L^{-1} BAP was most effective in the induction of the greatest number of adventitious shoots on white clover plants (Table 1). The BAP concentration of 0.5 mg L^{-1} was also an acceptable level to successfully micropropagate white clover. Statistical T-tests with these data showed the strongest statistical difference (P=0.05) when plants were exposed to 1.0 mg L^{-1} BAP (Table 2).

Table 1. Average number of shoots produced on clover plants that were grown in vitro on media with different levels of 6-Benzylaminopurine (BAP). Standard deviation (STDEV) and standard error (SE) are also shown.

	0 mg L^{-1}	**0.25 mg L^{-1}**	**0.5 mg L^{-1}**	**1.0 mg L^{-1}**
Mean	3.6	6.7	12.5	19.8
STDEV	1.2	5.2	12.7	12.9
SE	0.3	1.3	3.2	3.2

Table 2. T-test results on shoot production of clover plants growing on three different levels of 6-benzylaminopurine (BAP). P=0.05.

	25 mg L^{-1} BAP	***0.25 mg L^{-1} BAP***	**10 mg L^{-1} BAP**
0.0 mg L^{-1} BAP	0.0335	0.0136	0.0012
0.25 mg L^{-1} BAP		0.1.48	0.0012
0.5 mg L^{-1} BAP			0.1164

The information that was collected from this study with white clover allows for its implementation into a plan for the micropropagation of other clover plants. This was especially helpful for the clonal propagation of the new clover plants that were created as a result of this mutation breeding study (Figure 2). The in vitro cultivation of different species of *Trifolium* was performed in the same manner as white clover. The plants that were initiated and maintained in vitro using 1 mg L^{-1} BAP on MS medium were *T. incarnatum, T. wormskioldii, T. dichotomum* (syn. *amoenum*), and *T. pratense*. The endangered plant *T. dichotomum* also responded well in vitro. These results suggest that the methodology that was developed here can also be successful with all other species of *Trifolium*.

Surflan application

During the early stages of growth, all of the plantlets that were subjected to Surflan, except for the control plants, exhibited stunted growth. After 3 weeks, the 0.5% and 1.0% levels of Surflan killed at least 50% of the treated plants (Figure 3). An LD_{50}, or median lethal dose, is used as an indicator when mutations can be induced by a chemical. However, no physical mutations were observed in the plants exposed while in vitro. Later, as the plants grew to maturity, they expressed very few phenotypical mutations. Because not all mutations are phenotypically expressed, these results cannot guarantee that no mutations

occurred to these plants.

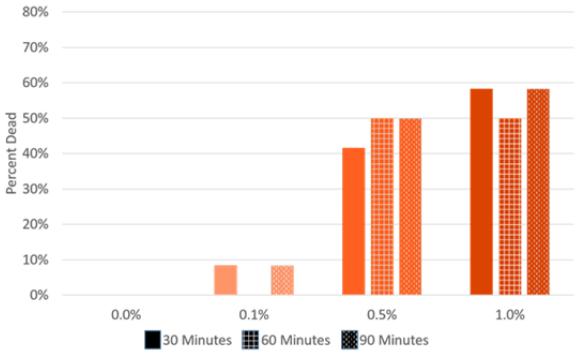

Figure 3. Survival rate of white clover plants 3 weeks after exposure that were subjected to three levels of Surflan for 30, 60, and 90 min.

Colchicine application

White clover plants that were subjected to 0.05% and 0.1% colchicine, whether for 48 h or 144 h, had greater than 50% mortality (Figure 4). As mentioned earlier, an LD_{50} is used as an indicator when mutations can be induced by a chemical. The plantlets that were exposed to colchicine at either doage time expressed minimal physical mutations in vitro. However, there was one physical mutation that produced a genetically stable six-leaf clover plant (Figure 5).

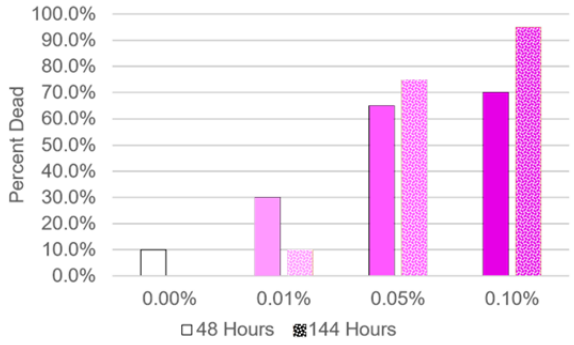

Figure 4. Survival rate of white clover plants 5 weeks after in vitro exposure to three concentrations of colchicine for 48 and 144 h.

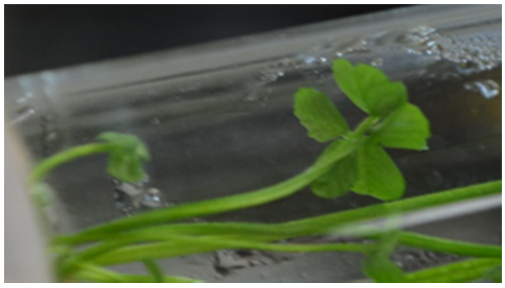

Figure 5. Colchicine-induced six-leaf white clover that was produced after being subjected to 0.1 g L^{-1} colchicine for 48 h in vitro.

Out of the two different approaches to mutagen exposures, the only one to produce a phenotypic mutation of significance was colchicine. The physical mutation induced was a six leaf clover (Figure 5). This mutation provides evidence that the 0.05 and 0.10% colchicine

concentrations in vitro were successful in the creation of a mutation in white clover with physical expression. It appears that the best LD_{50} concentration of colchicine lies between 0.01 and 0.05%.

CONCLUSIONS

The traditional propagation of clover plants by seeds was less efficient than their micropropagation in vitro. Traditional cultivation requires more resources, space, and handling time, whereas clover plants in vitro only require the confined space of a culture tube, the media in the tubes, and subculturing every 4-5 weeks. Propagation in vitro on media including the cytokinin BAP was more effective because a far greater number of clonal plants were able to be produced from one plant.

Propagation of clover plants by cross pollination produced genetically distinct plants and the mutation of clover plants with Surflan and colchicine also had limited success. The mutagens inflicted some degree of mutation to the plants that were exposed, but did not produce mutations that were aesthetically attractive. Plants that were subjected to Surflan had a stunting of growth and thickening of tissue. One attractive and interesting mutation, a multifoliate 6-leaf clover, was produced with colchicine.

Since the mutations that are induced by the chemicals are random, any aesthetically desirable trait being attained from the exposure would also be random. Further research with a greater number of plants would improve the chances of a successful mutation. Any unique plants that are produced, could then be propagated and bred for additional unique traits. This research has outline several techniques that can be used for this purpose.

Literature cited

Ascough, G.D., Staden, J.V., and Erwin, J.E. (2008). Effectiveness of colchicine and oryzalin at inducing polyploidy in *Watsonia lepida* N.E. Brown. HortScience *43*, 2248–2251.

Bae, T.W., Kang, S.Y., Lee, H.Y., Lim, P.O., and Song, I.J. (2009). WIPO Patent WO2009093787 A1.

Brickell, C., and Zuk, J. (1997). The American Horticultural Society A-Z Encyclopedia of Garden Plants (New York, New York: DK Pub).

Frame, J. (n.d.). *Trifolium repens* L. (Food and Agriculture Organization of the United Nations) http://www.fao.org/ag/agp/AGPC/doc/Gbase/data/pf000350.htm (Retrieved 20 Jan. 2014).

Hoveland, C.S., Hardin, D.R., Worley, P.C., and Worley, E.E. (1991). Steer performance on perennial vs. winter annual pastures in north Georgia. J. Prod. Agric. *4* (*1*), 24–28 http://dx.doi.org/10.2134/jpa1991.0024.

Jones, J.R., Ranney, T.G., and Eaker, T.A. (2008). A novel method for inducing polyploidy in Rhododendron seedlings. J. Amer. Rhododendron Soc. *62*, 130–135.

Kyte, L., Kleyn, J., Scoggins, H., and Bridgen, M. (2014). Plants from Test Tubes — an Introduction to Micropropagation, 4th edn (Portland, Oregon: Timber Press).

Lee, W.K. (2007). U.S. Patent No. 7230166 (Washington, DC, USA).

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. *15* (*3*), 473–497 http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Parente, G., and Frame, J. (1993). Alternative uses of white clover (FAO Corporate Document Repository) http://www.fao.org/3/a-v2350e/v2350e05.htm (Retrieved 13 Aug. 2014).

Quesenberry, K.H. (2002). Red, white, crimson, and other southern clovers: where have they gone and why? In Southern Pasture and Forage Crop Improvement Conference, 57th edn (Athens, Georgia: SPFCIC).

Schlesinger, N., Schumacher, R., Catton, M., and Maxwell, L. (2006). Colchicine for acute gout. Cochrane Database Syst Rev *4*, CD006190 10.1002/14651858.CD006190. PubMed

Søegaard, K. (1993). Nutritive value of white clover (FAO Corporate Document Repository) http://www.fao.org/docrep/v2350e/v2350e03.htm (Retrieved 13 Aug. 2014).

Stevens, D.R., Drew, K.F., Laas, K., and Turner, J.D. (1992). Deer production from ryegrass- and tall fescue-based pastures. Proc. N.Z. Grass Assoc. *54*, 23–26.