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Optimizing a Protocol for Long-term Storage of Duckweed Clones as Turions: Organization of Spirodela polyrhiza accessions in the RDSC

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Duckweed

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ABSTRACT

This protocol details about long-term storage of duckweed clones as turions. It contains protocols from the The International Steering Committee on Duckweed Research and Application (ISCDRA) Newsletter. A complete list of these news letters can be found here.

ATTACHMENTS

386-848.pdf

GUIDELINES

Introduction

Human has been collecting and maintaining plant germplasms since prehistoric time by the ways of seeds and living tissues for future use (Justice and Bass, 1978). Storage of plant germplasm is necessary to preserve and provide diverse genetic resources for crop improvement and is a primary target of conservation strategies (Migicovsky et al., 2019). Plant germplasms can be conserved through multiple methods such as seed banks, living collections, in vitro tissue/cell culture storage, and pollen banks (Laliberte, 1997). Unlike land plants, duckweeds are small aquatic plants growing on the surface of water bodies (Landolt, 1986). This aquatic angiosperm primarily multiplies through asexual propagation, which has limited its germplasm collection and conservation due to the labor and costs required in maintaining a living culture stock collection (Sree and Appenroth, 2020). In contrast, plant germplasm storage predominantly operates in the form of seed banks. Since viable seeds of most duckweed species are rare in either nature or the laboratory, duckweed clones are commonly maintained only as living collections. Large scale duckweed germplasm preservation was initiated by Elias Landolt at the Geobotanical Institute of the ETH Zurich, which stores well over 1000 clones at its peak (Sree and Appenroth, 2020). To date, facilities storing large duckweed collections exist in the USA, Germany, Switzerland, and China (Lam et al., 2020; Sree and Appenroth, 2020).

As vegetatively propagated species, duckweed clones can be stored in the laboratory for long periods via stock cultivation with periodic sub-culturing and maintenance (Sree and Appenroth, 2020). Duckweed germplasm stock collections are cultivated in various types of nutrient media, including N-medium with phosphate concentration increased to [M] 1 millimolar (mM), Steinberg's media with Fe- EDTA concentration increased to [M] 1 micromolar (μM), and Schenk and Hildebrand's (SH) medium (Appenroth, 2015). So far, two forms of culture medium have been used to maintain duckweed germplasm for large-scale collections: liquid and solid (e.g. agar). Maintaining duckweed germplasm in liquid solution offers a more close-to-natural habitat for duckweed growth. However, the main limitations are that this method requires more space, is a bit more expensive, and is more cumbersome to maintain for a large-scale collection. Nevertheless, liquid media could be a desirable option for increasing biomass and rescuing important germplasms, although our recommended standard for long term duckweed germplasm storage is to grow and transfer duckweed germplasm on agar plates at 15 °C (RDSC website; our survey in this issue of the DF). It has been reported that all duckweed clones collected from around the world are able to survive at temperatures of around 15-17°C (Sree and Appenroth, 2020).

Outlook

The use of turions as a storage vehicle to maintain some duckweed species could facilitate an alternative paradigm in storing duckweed germplasm. Here, we showed that turions of Sp. polyrhiza collected and stored using our protocol can readily germinate to produce new fronds and roots under de1ned medium and conditions. Using the dormant turion state may represent a more advanced method in the storage of duckweed germplasm, with the main advantages of lower labor and cost, need for fewer samples, and high viability as well as reliability of delivery. We believe this method can be used for ef1cient replacement of living collections of Sp. polyrhiza clones and we are currently taking steps to create turion stocks for the 100+ strains of this species in the RDSC. Turion storage may also be appropriate for some other duckweed species including almost all Wolf a species (with W. microscopica remaining unreported at present) and some Lemna species such as Lemna turionifera (Table 1). Thus, the standardization of turion production methods from these other species will be important next steps to follow by the RDSC. We hope to report on this effort to the community in the future.

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MATERIALS

Materials

- N-medium with phosphate
- Steinberg’s media with Fe-EDTA
- Schenk and Hildebrand's (SH) medium
- agar
- cefatoxime
- sucrose
- sodium hypochlorite
- KH_2PO_4
- $Ca(NO_3)2•4H_2O$
- KNO_3
- H_3BO_3
- $MnCl_2•4H_2O$
- Na_2MoO_4,
- $MgSO_4•7H_2O$
- Fe(III) EDTA
- baby jar
- Eppendorf tubes

Current protocol for clone maintenance in the RDSC

1

Note

The Rutgers Duckweed Stock Cooperative (RDSC) developed a general protocol for maintaining duckweed clones (Figure 1).

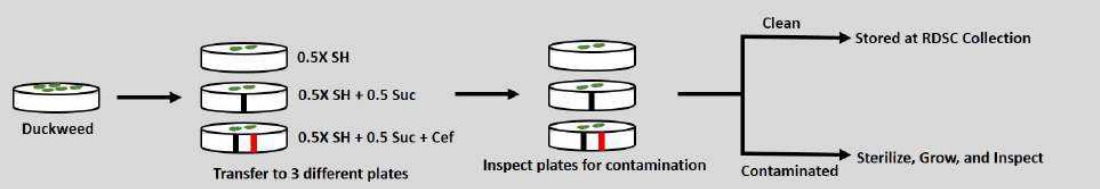


Figure 1. Scheme for duckweed stock maintenance. Healthiest duckweed fronds were selected and transferred to 3 plates
with different growth medium in 0.8% agar. After 2-3 weeks, the plates were inspected for contamination. Contaminated
duckweed were re-sterilized. Clean duckweed plates stored at RDSC collection.

Transfer approximately 5–10 duckweed fronds to three plates.

2 Use three types of plates comprising of 0.5X SH, 0.5X SH + Cef+ Suc 0.1%, or 0.5X SH +Suc 0.5% (Cef = cefatoxime; cf. Lam and Acosta, 2019).

> **Note**
>
> Essential nutrients and salts required by most duckweed are mimicked with 0.5X SH medium, while the antibiotic cefotaxime (Cef) is also present when indicated. This is used for backup plates, with the aim that the presence of cefotaxime could help control contaminations by bacteria (Lam and Acosta, 2019).

3 Addition of sucrose (Suc) at 0.1 or 0.5% (wt./vol.) can stimulate more rapid growth of fronds and visualize the presence of microbiological contaminations. The main limitation of storage using solid medium is the necessity of frequent plate examination to avoid early senescence and minimize overly rapid growth that can exhaust the nutrients in the plate.

4 Maintaining duckweed germplasm at lower temperatures and low light Euence (15 °C and 50–30 µmol m^{-2} s^{-1} white light) conditions can help to limit duckweed growth and delay senescence of duckweed clones (Sree and Appenroth, 2020) in order to extend the duration between each subculture steps and minimize the work load involved.

Using the inducible dormant state of duckweeds as a storag. 1d

5 As outlined in the steps, maintaining a substantial duckweed collection of over 100+ clones requires massive efforts to ensure the plants are kept in good health and minimize their loss due to contamination or accidental death. Trained staff play a key role in duckweed germplasm collection maintenance and curation; and stock cultures stored for long periods require routine observation to avoid nutrient depletion. Maintaining duckweed stocks on large scales is thus a complex and time-consuming effort. Therefore, it is necessary to develop more efficient and effective methods to maintain such collections and better serve the community.

6 To that end, we turn to the natural survival behavior that duckweeds and many other aquatic plants have evolved to deal with abiotic stresses. In unfavorable environments, such as low temperature and nutrient limitation, many duckweed species are known to alter the developmental program of their frond meristem to form a dormant structure that often detaches from the mother frond (Appenroth and Nickel, 2010).

7 These dormant forms of duckweed (ex. *Spirodela polyrhiza*), called turions, are round, dark green in color, and have an average diameter of 1–3 mm in diverse clones (Smart and Trewavas, 1983b).

8 Turions are also known to form in species from 11 genera of vascular aquatic plants (Adamec, 2018) and thus appears to be a fairly common strategy for overwintering in an aquatic environment. In the autumn, mature turions of Sp. *polyrhiza* sink to the bottom of water bodies in cold climates where they often stay unfrozen and await the return of appropriate conditions for germination and growth to resume in the spring (Smart and Trewavas 1983a).

9 Turion formation can also be readily induced under laboratory conditions by exposure to exogenous addition of the phytohormone abscisic acid (ABA), or through decreases in temperature and/or nutrients such as nitrogen, phosphate or sulfate (Appenroth and Nickel, 2010).

10 While it has been noted that turions can remain dormant and viable in the media that it formed in for quite some time (Landolt, 1986), to our knowledge it has not been systematically used as a strategy for clone maintenance in a stock collection scenario for any aquatic plants.

11 Through our project in the Lam lab to study the molecular basis of turion development, we became interested to translate our methods for turion induction and germination into a protocol for duckweed clone maintenance via turion production and storage.

12 Our approach to use turions for long-term storage of Sp. *polyrhiza*, the most well-studied duckweed species for turion biology, is outlined in Figure 2.

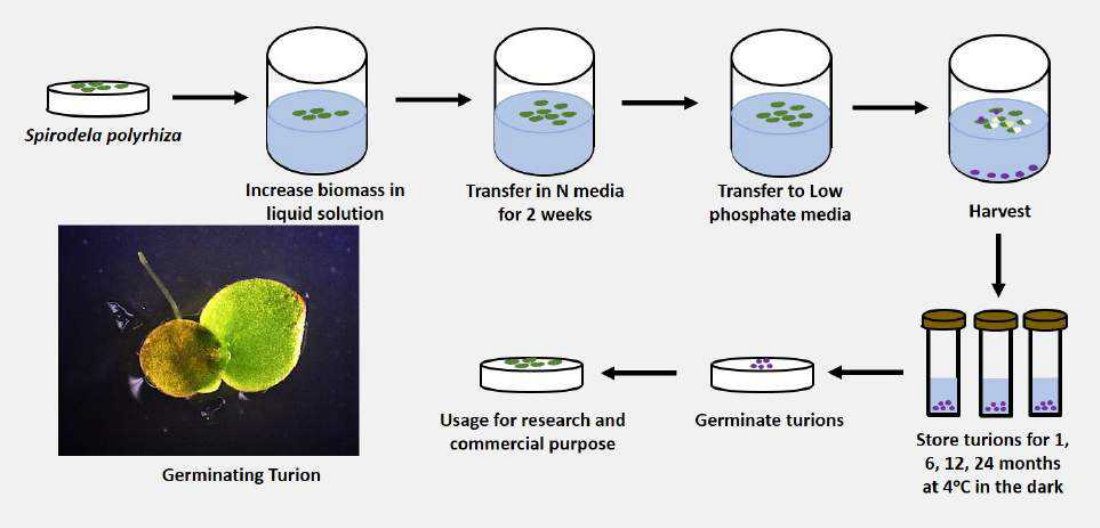


Figure 2. Turion induction and storage workflow. *Spirodela polyrhiza* biomass were increased by cultivating in 0.1% (W / V) sucrose containing liquid N-media under asceptic conditions. Biomass were then transferred to N-media only for two weeks before transferring to low phosphate N-media for 30 Days. Mature turions that have sunk to the bottom of the baby jars were harvested using a sterile disposable pipette. Turions are then stored in 2 ml Eppendorf tubes at 4ºC inside a covered box and tested for viability at different length of storage times. T: turion; F: germinated frond; R: germinated root

13 In this study, Sp. *polyrhiza* 9512 (Sp9512) and 9509 (Sp9509) clones were tested as well-characterized duckweed clones forming turion with high and low Specific Turion Yield (STY), respectively (Kuehdorf et al., 2014).

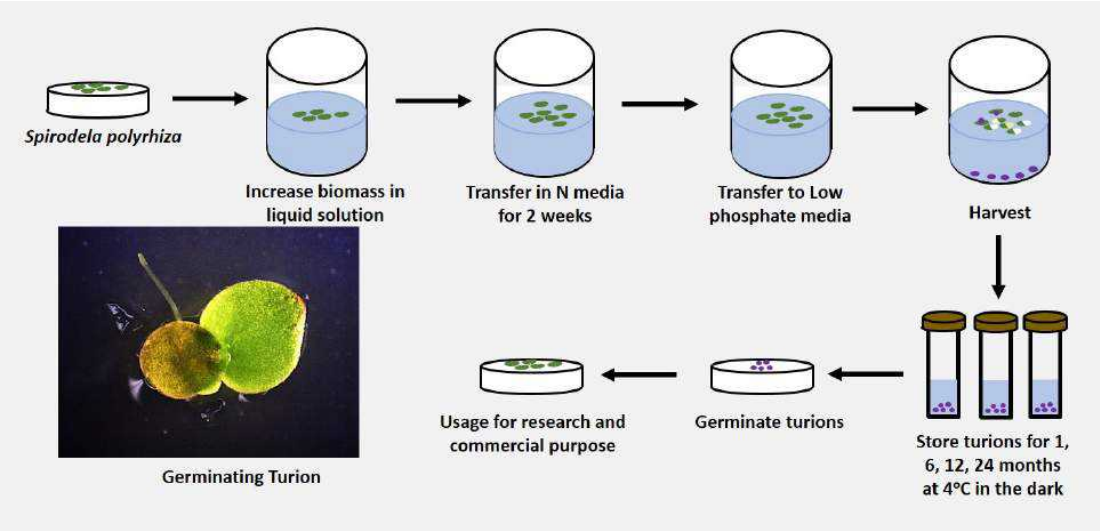
14 Grow two three-frond colonies of Sp9509 and Sp9512 from stock cultures in liquid media containing 0.5X SH medium with 0.1% (wt./vol.) sucrose for two weeks at 25 °C under illumination of 150 µmol m^{-2} s^{-1} light (16:00:00 light / 08:00:00 dark). 1d

15 Transfer 200 mg fresh fronds of each clones to a 177-ml baby jar containing 50 mL of N medium ([M] 60 micromolar (µM) KH_2PO_4, [M] 1 millimolar (mM) $Ca(NO_3)_2 \cdot 4H_2O$, [M] 8 millimolar (mM) KNO_3, [M] 5 millimolar (mM) H_3BO_3, [M] 13 micromolar (µM) $MnCl_2 \cdot 4H_2O$, [M] 0.4 micromolar (µM) Na_2MoO_4, [M] 1 millimolar (mM) $MgSO_4 \cdot 7H_2O$ and [M] 25 micromolar (µM) Fe(III) EDTA, pH 6.7) and grown for one more week under the same growth conditions.

16 To induce turion formation, transfer 300-500 mg fresh fronds of each clone to a 177-ml baby jar

containing 50 mL of N medium with low phosphate ([M] 2 micromolar (µM) KH_2PO_4, [M] 1 millimolar (mM) $Ca(NO_3)_2 \cdot 4H_2O$, [M] 8 millimolar (mM) KNO_3, [M] 5 millimolar (mM) H_3BO_3, [M] 13 micromolar (µM) $MnCl_2 \cdot 4H_2O$, [M] 0.4 micromolar (µM) Na_2MoO_4, [M] 1 millimolar (mM) $MgSO_4 \cdot 7H_2O$ and [M] 25 micromolar (µM) Fe(III) EDTA, pH 6.7). This phosphate concentration is the threshold value for several clones of Sp. *polyrhiza* to induce turion formation (Appenroth and Adamec, 2015).

17 Keep the inoculated fronds for more than 30 days under the same lighting and temperature conditions as before, until the full activation of turion production is observed, with mature turions accumulated at the bottom of the jar (Figure 2).



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18 Harvest these mature turions from the baby jars using a sterile disposable pipette and maintain in [M] 2 micromolar (µM) KH_2PO_4 (pH 6.7) solution.

19 Then, transfer 5–10 turions to 2 ml Eppendorf tubes containing 1.5 mL of N medium with low phosphate and store in the dark at 4 °C .

20 As they mature on the mother frond of Sp. polyrhiza, turions developed immature root and frond under their epidermis with thickened cell wall before they detach from the mother frond (Smart and Trewavas, 1983b).

21 After induction and formed under phosphate limitation, these turions remain dormant until after-ripening treatments with low temperature and appropriate light treatments before efficient germination can occur (Appenroth et al., 1989).

22 For the RDSC to use turions as an effective means of general storage of clones, it is necessary to examine the turion germination rate as a parameter to ascertain the viability of turions in our storage conditions and to discern potential clonal variations in this behavior.

23 To investigate turion germination rate post-storage, cultivate turions on 0.5X SH agar medium.

24 Remove the turions from the storage medium after various length of time in storage starting from 1 month post storage at 4 °C and sterilize surface using sodium hypochlorite solution (10%) followed by rinsing.

24.1 Rinse with sterile H_2O. (1/3)

24.2 Rinse with sterile H_2O. (2/3)

24.3 Rinse with sterile H_2O. (3/3)

25 Then, place 3–4 turions on an agar plate with 0.5X SH medium and observe for 14 days at 25 °C under illumination of 150 µmol m^{-2} s^{-1} light (16:00:00 light / 08:00:00 dark). 1d

26 Monitor the appearance of new fronds and roots as evidence for germination. Our study indicated that a new frond usually budded from the turion by day 2 post-germination followed by the first emerging root at day 3 to 4, and up to three developed fronds and multiple roots are observed by day 6 (Figure 3).

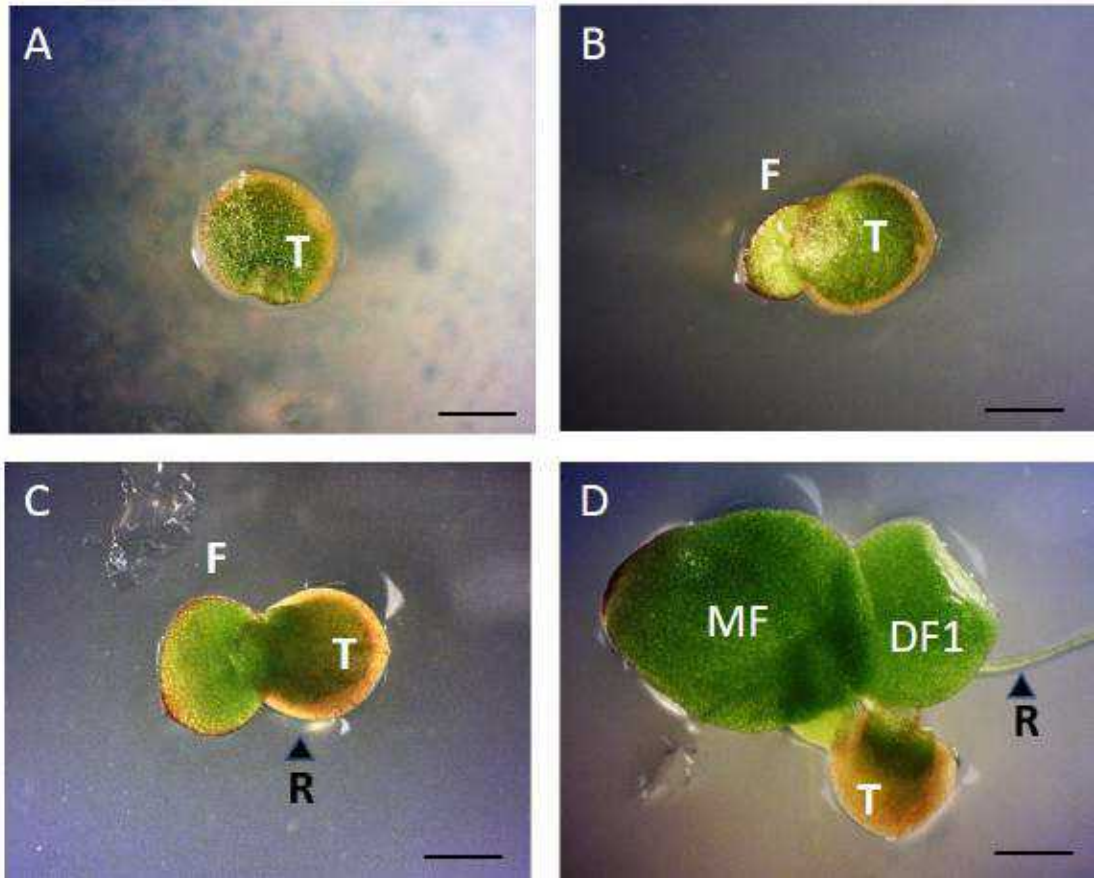

Figure 3. Stages of *Spirodela polyrhiza* 9512 turion germination. (A) Turion germinating in 0.5X SH solidified medium on Day 1. (B) After 2 days (Day 3), an expanding frond buds from the turion. (C) Day 4, the new frond grows out and an emerging root (black arrowhead) is observed. (D) Daughter fronds and root elongation observed at 6 days post germination. Note separation of frond cluster from the remain of the turion shell. Bar: 0.5 mm; T: turion, F: frond, R: root, MF: mother frond, DF1: first daughter frond.

27 Turion formation of different strains are known to be genetically adapted to a combination of multiple local climatic conditions including annual averages of temperature and precipitation or during the growing season (Kuehdorf et al., 2014).

28 We designed a controlled experiment to investigate the viability of turions from two Sp9512 and Sp9509 clones that were produced in parallel under identical conditions by initiating the low phosphate medium transfer about 1 week earlier with Sp9509 fronds than those of Sp9512.

29 Observe the turions to initiate around the same time for both clones and can be harvested on the same day under identical conditions and with the same buffers. After harvesting the mature turions of Sp9509 and Sp9512, store the turions for 1 month at 4 °C under dark conditions (after-ripening conditions) in order to break dormancy.

30 Investigate the germination rates of these mature turions to compare their viability under our conditions. Germination percentage was calculated using the formula
GP = (Total germinated turion)/(Total number of turion)*100.

31 Our results showed the germination rates for Sp9509 and Sp9512 turions in this trial are both 100%, thus indicating there is no significant difference in germination rates between these two Sp clones after 1 month of storage.

32 We will schedule to test these stored turions later on to compare their viability over longer periods (e.g. 6 months, 1 year) to ascertain further the length of time that they could be stored.

33 As previous experience with long term storage of turions from another Sp. *polyrhiza* strain 9500 has observed good viability after 5 years at 4 °C (Appenroth, personal communication), we are optimistic that these turions should remain viable as well for at least up to a year.

Planned curation protocol for RDSC service

34

Note

We report here a protocol that should enable us to reliably induce and harvest turions from duckweed strains. In the present study, turions were successfully induced in Sp. *polyrhiza* clones by simply lowering the phosphate concentration in the growth medium as a test case for storage. Leveraging this protocol, we could also test this method in the future for duckweed clones from the genus Wolf a as well as the species *Lemna turionifera* and *Le. aequinoctialis* (Table 1) that have been known for their ability to form turions (Landolt, 1986, Les et al., 1997).

	A	B	C
	Genus	Species	Distribution
	Lemna	Lemna aequinoctialis	Africa, Asia, Europe, North America, Central America, and South America
		Lemna turionifera	Asia, Europe, and North America
	Spirodela	Spirodela polyrhiza	Africa, Asia, Australia, Europe, North America, and South America
	Wolffia	Wolffia angusta	Asia, and Australia
		Wolffia arrhiza	Africa, Europe, and South America
		Wolffia australiana	Australia, and Paci1c
		Wolffia borealis	Asia, and North America
		Wolffia brasiliensis	North America, Central America, and South America
		Wolffia columbiana	North America, and South America
		Wolffia elongata	Asia, and South America
		Wolffia globosa	Asia, North America, and South America

Table 1. List of *Lemnaceae* species observed to form turions (Landolt, 1986, Les et al., 1997)

The objective of sharing our curation protocol is to provide guideline to help reduce operating cost and time-consuming effort when managing a substantial number of clones in these species that are able to form turions. We hope this standardized method can be applied to future collection efforts to improve the service provided by RDSC as well as by other collections. Its implementation should also simplify the effort for clone distribution pipeline to improve reliability for delivery and better viability of duckweed germplasm.

As an example, store 5 Sp. *polyrhiza* turions in 1.5-ml Eppendorf tubes containing 1 mL of mineral salt medium with low phosphate at 4 °C in the dark. Dark and cold condition is likely to mimic the natural condition during overwintering.

35 Label the stored turions based on their clone number.

36 As an order is received for this clone, surface sterilize the turions using sodium hypochlorite solution (10%) followed by rinsing.

36.1 Rinse the turions with sterile H_2O. (1/3)

36.2 Rinse the turions with sterile H_2O. (2/3)

36.3 Rinse the turions with sterile H_2O. (3/3)

37 Then the turions will be germinated on agar plates and should be ready to be sent to the requester within about 1-2 week's time.

38 The key advantage of this method is the avoidance of having to repeatedly subculture the 100+ strains of Sp. *polyrhiza* clones that are currently in the RDSC stock collection.

39 Furthermore, it should minimize the variability of conditions that each stock culture may be in at the time of ordering, thus giving a more reliable delivery time upon receipt of orders.

40 Extending this method of germplasm maintenance to other turion-forming duckweed species in the future should further decrease the work-load for the RDSC while minimizing loss of culture stocks from contamination or senescence.