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Micropropagation of Prickly Pear by Axillary Shoot Proliferation

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[Abstract] A protocol for the axillary bud proliferation of prickly pear (*Opuntia; Cactaceae*) is presented. This genus is widely used as a crop in the arid and semi-arid areas of the globe worldwide, providing numerous benefits for human and animal consumption. *In vitro* culture for axillary bud proliferation is of great use to obtain a large quantity of plants in a short period of time, with potential uses in production and for the preservation of endangered species of the *Opuntia* genus.

The optimal medium for *Opuntia in vitro* culture consists of Murashige and Skoog medium (MS) and L2 vitamins. To increase the yield of the axillary bud proliferation, we recommend the addition of plant growth regulators (PGRs). This work suggests a 15 d incubation in the medium with 2.2 mg/L of benzyl aminopurine (BA) after which the explants are transferred to the medium without PGRs. We explain as well how to adapt the plant to *ex vitro* conditions.

Keywords: Prickly pear, *In vitro* culture, Axillary bud proliferation, *Ex vitro* adaptation

[Background] The genus *Opuntia* (prickly pear) is one of the members of the *Cactaceae* family (Bravo-Hollis, 1978). Although it is native to the Americas, it currently grows in the wild and commercial plantations of the South of Europe, North of Africa, Australia, Middle East, West Asia and other regions of the world (Ochoa and Barbera, 1995; Kiesling and Metzing, 2017). Prickly pear has profound effects on the arid and semi-arid environments as well as the human communities that live in those areas because of its high biomass yields despite growing in drylands (Acevedo *et al.*, 1983). In many of such areas, the genus *Opuntia* is exploited for a number of things. The young cladodes of *Opuntia* can be consumed as a vegetable; the fruit is eaten directly or processed into jelly, juice or sweets (Barba *et al.*, 2017). The whole plant can be used for animal forage, living fence (Las Casas *et al.*, 2017) and recently it started to be used commercially to produce biofuel (Aké Madera, 2014). In addition, the *Opuntia* genus has a number of bioactive and nutritionally valuable compounds (Betancourt *et al.*, 2017; Melgar *et al.*, 2017).

The advantages of *in vitro* culture include high proliferation rates and the production of pathogen-free plants (Shedbalkar *et al.*, 2010). In their native environments, some *Opuntia* species are suffering losses in their populations, due to exploitation without replacement that has been carried out traditionally (Rocha-Flores *et al.*, 2017). *In vitro* culture thus brings the possibility of recovering the populations of endangered plants (Torres-Silva *et al.*, 2018), as well as a large scale production for agriculture. Here we present a protocol for plant *in vitro* culture of the *Opuntia* genus that includes the cytokinin benzyl aminopurine (BA).

Materials and Reagents

1. 1,000 glass culture containers (100 ml) (Sigma-Aldrich, catalog number: B8648)
2. Parafilm
3. Scapel
4. Petri dish
5. Nursery trays (International GreenHouse, catalog number: CN-PLG)
6. Humidity dome (International GreenHouse, catalog number: PR-DOME7)
7. An *Opuntia sp.* young tender cladode
8. Dishwashing liquid
9. Deionized water
10. 96% alcohol
11. 60% sodium hypochlorite (NaClO) (household bleach)
12. 1 N NaOH
13. 1 N HCl
14. Tween 20 detergent
15. Benzyl aminopurine (6-benzyl aminopurine) (Sigma-Aldrich, catalog number: B3408)
16. Activated charcoal (Sigma-Aldrich, catalog number: C9157)
17. Agar-agar (Sigma-Aldrich, catalog number: A1296)
18. Sucrose ($C_{12}H_{22}O_{11}$) (Sigma-Aldrich, catalog number: S5391)
19. Ammonium nitrate (NH_4NO_3) (Sigma-Aldrich, catalog number: A3795)
20. Potassium nitrate (KNO_3) (Sigma-Aldrich, catalog number: NIST193)
21. Calcium chloride dihydrate ($CaCl_2·2H_2O$) (Sigma-Aldrich, catalog number: 223506)
22. Magnesium sulfate heptahydrate ($MgSO_4·7H_2O$) (Sigma-Aldrich, catalog number: 1374361)
23. Potassium dihydrogen phosphate (KH_2PO_4) (Sigma-Aldrich, catalog number: PHR1330)
24. EDTA (Na_2EDTA) (Sigma-Aldrich, catalog number: PHR1068)
25. Ferrous sulfate heptahydrate ($FeSO_4·7H_2O$) (Sigma-Aldrich, catalog number: 1270355)
26. Manganese sulfate tetrahydrate ($MnSO_4·4H_2O$) (Sigma-Aldrich, catalog number: 229784)
27. Zinc sulfate ($ZnSO_4·7H_2O$) (Sigma-Aldrich, catalog number: Z0251)
28. Boric acid (H_3BO_3) (Sigma-Aldrich, catalog number: B6768)
29. Potassium iodide (KI) (Sigma-Aldrich, catalog number: 746428)
30. Sodium molybdate dihydrate ($Na_2MoO_4·2H_2O$) (Sigma-Aldrich, catalog number: M1003)
31. Cupric sulfate pentahydrate ($CuSO_4·5H_2O$) (Sigma-Aldrich, catalog number: C3036)
32. Cobalt chloride hexahydrate ($CoCl_2·6H_2O$) (Sigma-Aldrich, catalog number: C2911)
33. Myo-inositol ($C_6H_{12}O_6$) (Sigma-Aldrich, catalog number: I7508)
34. Thiamine hydrochloride ($C_{12}H_{17}ClN_4OS·HCl$) (Sigma-Aldrich, catalog number: T1270)
35. Pyridoxine ($C_8H_{11}NO_3$) (Sigma-Aldrich, catalog number: P5669)
36. MS + L2 vitamins salts (see Recipes)
37. Disinfection solution (see Recipes)

38. Maintenance medium (see Recipes)
39. Stimulation medium (see Recipes)
40. Rooting medium (see Recipes)
41. Substrate for *ex vitro* adaptation (see Recipes)

Note: All reagents are stored at room temperature and have a long shelf life (~10 years). Maintenance, stimulation and rooting media can be stored up to 15 d at 4 °C. Disinfection solution should be prepared and used the same day.

Equipment

1. Tweezers
2. Microwave oven
3. pH meter
4. Weighing balance
5. Autoclave
6. Laminar flow hood
7. Greenhouse
8. Growth chamber

Procedure

A. Preparative steps
   1. Prepare 2 L 10x MS Stock medium (see Recipes).
   2. Use the 10x MS Stock medium to prepare 8 L of maintenance medium, 2 L of stimulation medium, and 10 L of rooting medium (see Recipes). These quantities are calculated to obtain approximately 1,000 plants to be transferred to *ex vitro* conditions. The number of shoots sprouting from the explants is highly variable, so it must be taken into consideration that this number is not precise.
   3. Melt the maintenance, stimulation and rooting media in a microwave oven, aliquot 25 ml into each glass culture container and autoclave. Alternatively, autoclave the media and aliquot into sterile glass culture containers under a flow hood.
   4. Autoclave 20 glass culture containers to be used for explant disinfection (Step B4).

B. Establishment of the *in vitro* culture
   1. Cut a young, tender cladode from the parent plant (Figures 1A and 1B). Young cladodes should be 3-4 weeks old after sprouting from the mother cladode and 5-15 cm long.

Figure 1. Source of plant material. A. 18 months-old plant of *Opuntia ficus-indica* with a young tender cladode (white frame). B. Young tender cladode suitable for starting micropropagation. The black arrow points at an areole, the structure where new buds will develop.

2. Wash the young cladode gently with running water and dishwashing liquid to eliminate dirt.
3. Divide the cladode in sterile conditions into four pieces (Figure 2). Meristems, crucial for this micropropagation method, are regularly spaced throughout the cladode in structures called areoles (Figure 1B). It must be made sure that every fragment of the cladode contains at least 5 areoles for the proliferation to be successful.

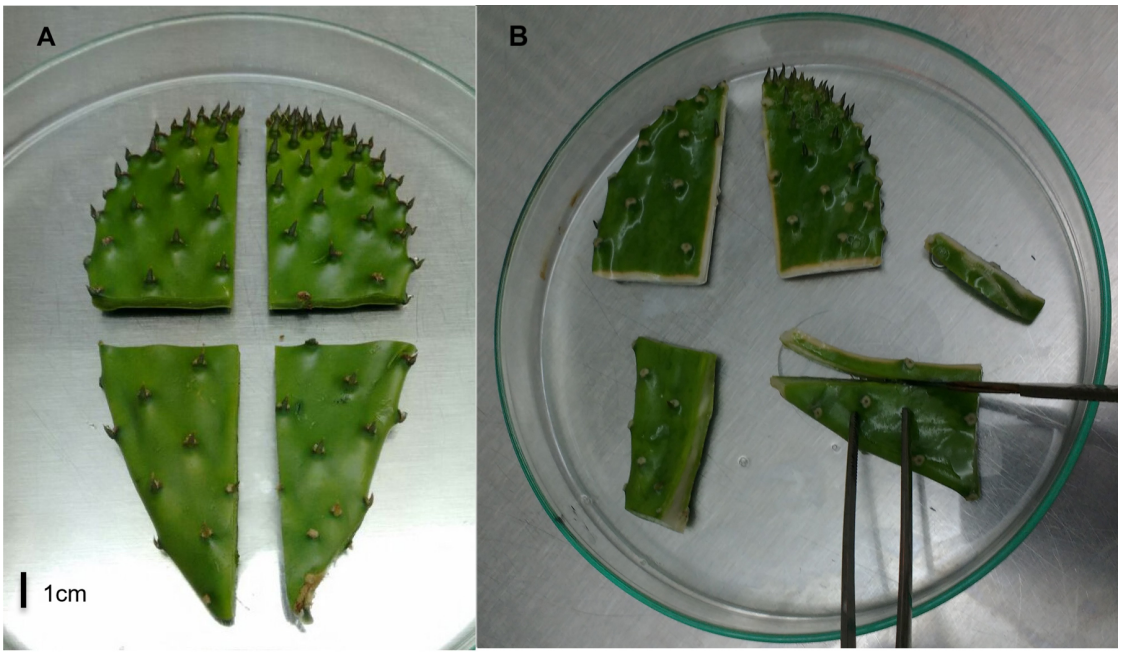

Figure 2. Explants of young tender cladode before (A) and after disinfection (B). Damaged tissue, with a yellowish appearance, must be removed before placing the explant in maintenance medium.

4. For each explant, prepare 5 sterile containers in the following way under a laminar flow hood:
   a. Fill one container with 96% alcohol, enough to fully cover the cladode.
   b. Fill one container with 60 ml disinfection solution (see Recipes).
   c. Fill three containers with 60 ml sterile deionized water.
5. Immerse the cladode into 96% alcohol for 10 sec.
6. With the aid of sterile tweezers, transfer the cladode to disinfection solution and shake gently for 10 min.
7. Place the cladode in a container with 60 ml of sterile deionized water. Shake gently for 1 min.
8. Repeat the previous step two more times, changing the cladode each time to a new container.
9. Place the cladode into a sterile Petri dish.
10. The disinfection will probably damage the exposed tissue left by the cutting. Thus, remove the damaged tissue with a scalpel.
11. Transfer the pieces and place them into the solid maintenance medium (see Recipes).
12. The pieces must be half buried (Figure 3). It is important that the exposed tissue is in contact with the medium to facilitate the absorption of nutrients by the cladode.

Figure 3. Position in which the cladode explants should be placed in the maintenance medium

13. Place in a growth chamber with a 16 h light/8 h darkness photoperiod at a light intensity of 25 µmol photons m^{-2} sec^{-1}, at 25 °C.
14. Monitor frequently the explants. The meristems will be activated and start growing. One to four weeks after having established the explants, an approximately 2 cm shoot will have grown from at least one of the meristems (Figure 4). If contamination appears, discard those containers and continue only with the non-contaminated. If oxidation appears, parts of the explant turning dark brown, remove the damaged part and place in a new container with medium.

Figure 4. Shoot derived from the initial explant after stimulation placed on maintenance medium

C. Proliferation of plant material

1. New shoots would have developed and grown during the establishment phase. Cut these new shoots from the explant at 0.5 cm from the base on a Petri dish under sterile conditions.
2. Transfer the new shoots to a container with stimulation medium (see Recipes) with the apical meristem facing up. The growth regulator in the medium will stimulate axillary shoot proliferation from the basal meristems. It is important that no more than four explants are together in the same container. Placing more will hamper their growth as they would compete for nutrients.
3. After 20 days, a variable number of new shoots will have developed from the initial shoot (Figure 5). Take the whole explant and transfer to maintenance medium.

Figure 5. Shoots derived from shoot explants cultivated in stimulation medium

4. After 2 months, the shoots derived from axillary shoot proliferation will reach the size of 3 cm. The axillary shoots do not proliferate synchronously. Wait until most of the shoots have this length and divide under sterile conditions into 1.5 cm explants. Ignore if some small roots already developed.
5. At this point, new shoots would have grown from the explants. Repeat Steps C2-C4. Shoots derived from explants that already passed a round of stimulation/maintenance usually have a different morphology in comparison to the first shoots derived from cladode segments (Figure 6). At this point, an estimated 1,000 plants will have been produced, for which the amount of media suggested to prepare will be enough. If plants need to be propagated further, each new round of Steps C2-C4 will increase the yield of plants by a factor of 15.

Figure 6. Shoot derived from shoot explants after a second round of Steps C2-C4 of 'Proliferation of plant material'

D. *Ex vitro* establishment of plant material

1. After several rounds of maintenance/stimulation and the desired amount of material is obtained, 4-5 cm shoots are cut and placed into rooting medium (Figure 7).

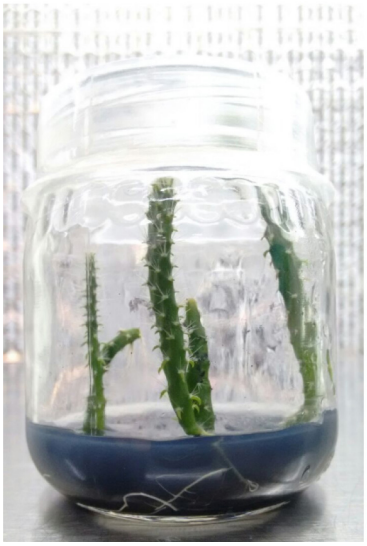

Figure 7. Shoot explants in rooting medium 3 weeks after transfer

2. Monitor the explants for the next 2 months. After this long period, roots will develop. At the same time, the medium will lose moisture, which hardens the plant and prepares it for *ex vitro* conditions (Figure 8A). Characteristics that are typical of cacti will appear at this point, such as a thick cuticle. Small spines develop, and the surface acquires a dark-green color (Figure 8B).

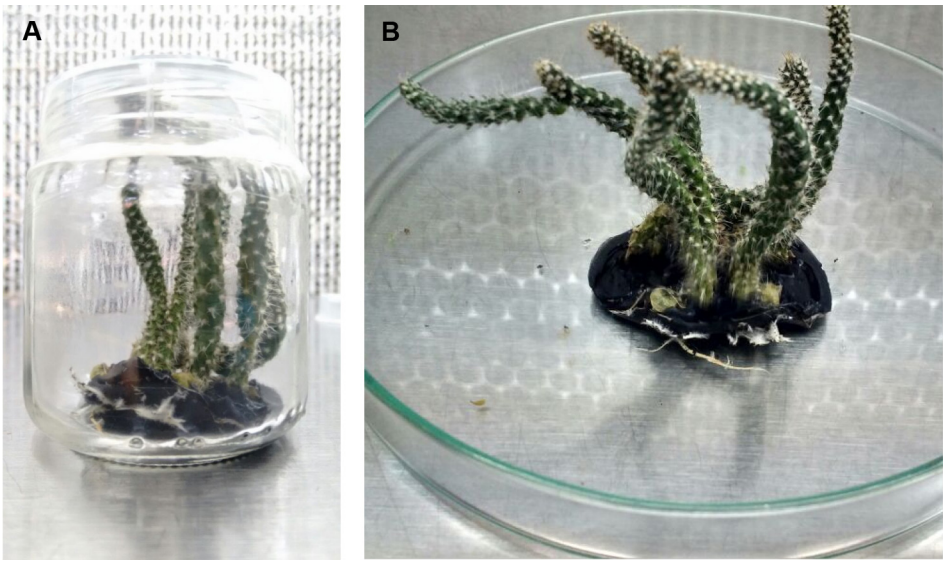

Figure 8. Plants ready for *ex vitro* adaptation inside (A) and out of the container (B). The medium looks already dehydrated and the plant hardened. The root development is essential for its survival under *ex vitro* conditions.

3. Warm 50 ml tap water in the microwave up to 40 °C.
4. Open the containers, and take out the explants.
5. Rinse the plant roots with warm water to remove the remaining solid medium.
6. Add substrate for *ex vitro* adaptation (see Recipes) to the nursery trays.
7. Water the substrate in the nursery trays with excess water, when the trays stops draining, the substrate will be at field capacity, and ready to use.
8. Place the plant carefully into a pot with substrate (see Recipes) at field capacity. The plant should be placed vertically and approximately 1 cm of the stem must be under the surface of substrate, to completely cover the roots.
9. Cover the nursery trays with humidity domes and keep for 2 weeks in a greenhouse. The hood will keep a moist atmosphere (approximately 80% relative humidity). This step is essential. Even though *Opuntia* is natural to dry areas, *in vitro* grown plants keep their stomata open and are highly prone to dehydration. In tropical and subtropical areas of the world, natural light can be used. In lower latitudes, artificial lighting at 180 to 200 µmol photons m^{-2} sec^{-1} is recommended.
10. In the course of one week, remove the plastic hood and leave the nursery tray open for 2 h every day. It is important to keep the soil at field capacity during this whole period.
11. For another period of one week, increase the exposure to air to 4 h daily. As in the previous step, keep substrate at field capacity. This gradual exposition to the air will allow the plant to fully adapt to *ex vitro* conditions.
12. If plants are to be kept in the greenhouse for their whole lifetime, the protocol can end here. *Opuntia* plants need a light intensity between 750 and 1,700 µmol photons m^{-2} sec^{-1}, at lower light intensities plants would over-elongate and have an atypical appearance.
13. If plants are to be transferred to field conditions they must be placed for 3 more months in a

plant nursery (Figure 9), where they can be kept away from diseases, weeds and water stress so they develop healthily and maximize the chances of survival in the field.

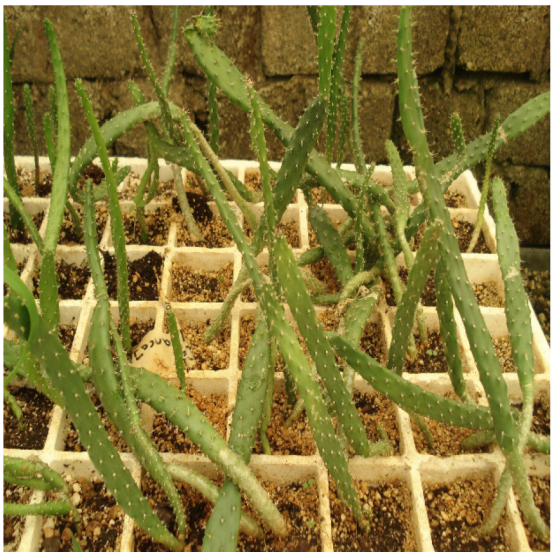

Figure 9. Plants at the end of the protocol after 3 months in a plant nursery

Notes

1. This protocol is written generically for the micropropagation of any species of the *Opuntia* genus. The protocol was developed initially for *Opuntia ficus-indica*, but has also shown to be effective for *O. atropes*, *O. cochenillifera*, *O. jaliscana*, *O. joconostle*, *O. robusta*, *O. schickendantzii* and *O. tunicata.*
2. During the establishing phase, the explant could be put directly into stimulation medium instead of maintenance medium. Nevertheless, there is a risk that the material undergoes oxidation. This is highly dependent on the genotype.
3. Oxidation can hinder the establishment of plant material under *in vitro* conditions even with medium without PGRs. In this case, anti-oxidant additives can be added to the maintenance medium such as ascorbic acid (125-250 mg/L), citric acid (125-250 mg/L) and polyvinylpolypyrrolidone (PVP) (1-3 g/L). Ascorbic acid and citric acid can be used together. PVP should be used alone.
4. The number of shoots obtained during the stimulation phase and the speed at which they elongate highly depends on the genotype.

Recipes

1. 10x MS + L2 Stock medium (1 L)
   a. MS salts (Murashige and Skoog, 1962)
      Sucrose ($C_{12}H_{22}O_{11}$) 300 g

Ammonium Nitrate (NH_4NO_3)	16.5 g
Potassium Nitrate (KNO_3)	19 g
Calcium Chloride ($CaCl_2·2H_2O$)	4.4 g
Magnesium Sulfate ($MgSO_4·7H_2O$)	4.0 g
Potassium Dihydrogen Phosphate (KH_2PO_4)	1.7 g
EDTA (Na_2EDTA)	373 mg
Ferrous Sulfate ($FeSO_4·7H_2O$)	278 mg
Manganese Sulfate Tetrahydrate ($MnSO_4·4H_2O$)	223 mg
Zinc Sulfate ($ZnSO_4·7H_2O$)	107 mg
Boric Acid (H_3BO_3)	62 mg
Potassium Iodide (KI)	8.3 mg
Sodium Molybdate Dihydrate ($Na_2MoO_4·2H_2O$)	2.5 mg
Cupric Sulfate ($CuSO_4·5H_2O$)	0.25 mg
Cobalt Chloride ($CoCl_2·6H_2O$)	0.25 mg

b. L2 vitamins (Phillips and Collins, 1979)

Myo-inositol ($C_6H_{12}O_6$)	2.5 g
Thiamine hydrochloride ($C_{12}H_{17}ClN_4OS·HCl$)	20 mg
Pyridoxine ($C_8H_{11}NO_3$)	5 mg

c. Dissolve all of the previous reagents except Na_2EDTA and $FeSO_4$ in 500 ml deionized water

d. Dissolve Na_2EDTA and $FeSO_4$ in 30 ml warm deionized water, and then add to the previous solution

e. Make up to 1 L with deionized water

Note: We recommend dividing the stock into 100 ml aliquots and freezing to avoid growth of microorganisms.

2. Disinfection solution (60 ml)

   30 ml sterile deionized water

   30 ml household bleach

   3 drops of Tween 20 detergent

   Prepare under sterile conditions

   Mix well to avoid the detergent to fall to the bottom of the solution

3. Maintenance medium (1 L)

   900 ml sterile deionized water

   100 ml 10x MS + L2 stock medium

   8 g agar-agar

   Mix the sterile deionized water with the 10x MS + L2 stock medium

   Adjust pH to 5.8 ± 0.05 with 1 N NaOH or 1 N HCl

   Add agar-agar

   Autoclave at 1.3 kg/cm^2, at 120 °C for 15 min

4. Stimulation medium (1 L)
   900 ml sterile deionized water
   100 ml MS + L2 stock medium
   2.2 mg BA
   8 g agar-agar
   Dissolve BA in a few drops of 1 N NaOH
   Add sterile deionized water until reaching 2.2 ml
   Add 10x MS + L2 stock medium
   Make up to 1 L with deionized water
   Adjust pH to 5.8 ± 0.05 with 1 N NaOH or 1 N HCl
   Add agar-agar
   Autoclave 1.3 kg/cm^2, at 120 °C for 15 min
5. Rooting medium (1 L)
   900 ml deionized water
   100 ml 10x MS + L2 stock medium
   2 g activated charcoal
   8 g agar-agar
   Mix the sterile deionized water with the MS + L2 stock medium
   Adjust pH to 5.8 ± 0.05 with 1 N NaOH or 1 N HCl
   Add agar-agar
   Autoclave 1.3 kg/cm^2, at 120 °C for 15 min
6. Substrate for *ex vitro* adaptation (50 L)
   30 L sand (1.7-4.75 mm particle diameter)
   15 L peat moss
   5 L composted manure
   Prepare a 6:3:1 (v/v) mixture of sand:peat moss:manure
   Note: This quantity is enough to fill eight nursery trays of 60 holes each.

Acknowledgments

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