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In vitro growth profile and comparative leaf anatomy of the C_3–C_4 intermediate plant *Mollugo nudicaulis* Lam.

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Abstract
Mollugo nudicaulis Lam., commonly known as John's folly or naked-stem carpetweed, is an ephemeral species of tropical regions. The plant is ideal to study the eco-physiological adaptations of C_3–C_4 intermediate plants. In the present report, *in vitro* growth profiling of the plant and comparative leaf anatomy under *in vitro* and *ex vitro* conditions were studied. *In vitro* propagation of the plant was carried out on Murashige and Skoog (MS) basal medium augmented with additives and solidified with 0.8% (*w/v*) agar-agar or 0.16% (*w/v*) Phytagel™. The concentration of plant growth regulators (PGRs) in the basal medium was optimized for callus induction, callus proliferation, shoot regeneration, and *in vitro* rooting. The optimum callus induction was obtained from *M. nudicaulis* seedling hypocotyls. The highest regeneration induction of about 88% or nearly 41 shoots with about 142 leaves per culture vessel was observed from friable callus on MS basal medium solidified with Phytagel™ and containing 4.44 μM 6-benzylaminopurine, 4.65 μM kinetin, 2.69 μM naphthaleneacetic acid, and 0.91 μM thidiazuron. In leaf anatomy, differences related to photosynthetic tissue organization were observed in leaves of *in vitro* and *ex vitro* plants, which indicated that changes in the environment affected the anatomy of subsequent leaves in plants. This is the first report of an efficient micropropagation protocol for *M. nudicaulis*, using an indirect organogenesis method. Efforts were made to optimize the concentrations of various PGRs and organic compounds for *in vitro* growth of regenerated shoots.

Keywords Micropropagation · Tropical C_3–C_4 intermediate · Phytagel · Gibberellic acid · Aeroponics

Introduction

The C_3 and C_4 systems are well-known pathways of photosynthesis that assimilate the carbon dioxide (CO_2) from the air in green leaf cells. By their physio-ecological adaptation to high temperature and low availability of water, nitrogen, and CO_2, plant species with the C_4 photosynthesis system are comparatively more advanced and efficient than C_3 species (Rawsthorne 1992 ; Christin and Osborne 2014; Stata *et al.* 2014). Besides C_3 and C_4 plants, there are certain plant species that have CO_2 compensation points between those of C_3 and C_4 plants and are thus known as intermediates or C_2 types (Rawsthorne 1992; Sage *et al.* 2014; Schlüter and Weber 2016). Such C_3–C_4 intermediate species are geographically rare in comparison to both C_3 and C_4 species (Nicholson 2011; Christin and Osborne 2014). These C_3–C_4 intermediate plants may have evolved to adapt to climatic conditions interlinking C_3 and C_4 species in the same genus or may have a separate origin (Voznesenskaya *et al.* 2010; Gowik and Westhoff 2011). At present, C_3–C_4 intermediate species have been reported in different clades of 17 genera of flowering plants, with one to two species in each genus (Sage *et al.* 2011). To date, only two C_3–C_4 intermediate species with a cosmopolitan distribution in warm climates, *Mollugo nudicaulis* Lam. and *Mollugo verticillata* L., have been reported (Vincent 2003). These plants are suitable for studying the function of warm climate C_3–C_4 intermediates.

In a desert climate, a variety of plant species are ephemeral and complete their life cycle during the rainy season (Nicholson 2011). These plants are adapted to either high or low temperatures at anatomical, biochemical, and physiological levels. According to The International Plant Names Index 2012, *Mollugo nudicaulis* Lam., also known by its synonym *Paramollugo nudicaulis* (Lam.) Thulin (Thulin *et al.* 2016), is

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an ephemeral species, belonging to the family Molluginaceae, order Caryophyallales (Bhandari 1990; Sukhorukov and Kushunina 2016). The plant possesses a characteristic feature of rosette leaves with many inflorescence stalks (Fig. 1*a–c*). This species is characterized as a C_2 type I intermediate, with low levels of C_4 metabolism (Christin *et al.* 2011; Sage *et al.* 2014). Additionally, *M. nudicaulis* possesses hepatoprotective properties (Rajamanikandan *et al.* 2012), and its extract is also used to cure eye and skin infections, whooping cough, and typhoid in traditional medicine (Ignacimuthu *et al.* 2008; Rameshkumar and Sivasudha 2012).

The development of micropropagation protocols for plants helps in conserving the germplasm and provides disease-free, synchronized plant material in a short time (Mosaleeyanon *et al.* 2004). Additionally, these techniques can constitute an essential first step toward effective transgenic plant engineering and crop manipulation (Jauhar 2006) and an informative means for studying gene function (Thole and Rawsthorne 2003). Nagesh and Shanthamma (2011) reported a preliminary study regarding the micropropagation of *M. nudicaulis* using shoot tip explants and explored for its antioxidant properties.

The present paper analyzes the *in vitro* growth profile of *M. nudicaulis* to achieve effective regeneration of plants throughout the year. During *in vitro* conditions, plants are subjected to a saturated environment, which often leads to the modification of anatomical features. These phenomena could be exploited for studying the comparative leaf anatomy of these C_3–C_4 intermediate plants at *in vitro*, as well as *ex vitro*, levels. This is the first report of a comparative study on anatomical intermediate characteristics found in *ex vitro* and *in vitro*-grown leaves of *M. nudicaulis*.

Materials and methods

Plant material Whole plants of *M. nudicaulis* were collected at flowering and fruiting stages in early August to late October from natural and cultivated areas in Jodhpur, India (26.350932 N, 73.046042 E). These plants were grown under greenhouse conditions in an aeroponic chamber made up of styrofoam sheets according to Mehandru *et al.* (2014), where moisture was maintained through water mist applied to the basal parts of the plants at regular intervals of 60-s misting with an 800-s pause between consecutive misting. In this setup, misting of water on basal parts of roots was carried out through nine 50-μ-high pressure nozzles connected to 50-mm diameter polyvinyl chloride (PVC) pipes (Prince piping system, Mumbai, India) through which 125 mL of water per nozzle was pumped by a 0.373-kW electric motor (Crompton Greaves, Mumbai, India) at a pressure of 4.21 kg cm^{-2}.

To establish the *in vitro* culture, two different types of explant were selected, juvenile explants and mature leaves. Seeds and mature leaf tissue were surface sterilized with 0.1% (*w/v*) Bavistin® (a fungicide containing 50% (*w/w*) Carbendazim, BASF India Limited, Mumbai, India) for 10 min, washed three to four times with autoclaved, distilled water, and then sterilized with 0.1% (*w/v*) $HgCl_2$ (Sigma-

Figure 1. Depiction of *in vitro* responses of *Mollugo nudicaulis* Lam. (*a*) *Ex vitro* plant in its natural habitat; (*b*) flower; (*c*) mature seeds; (*d*) *in vitro* seed germination; (*e*) callus induction (indicated by *arrow*) from hypocotyl; (*f*) callus proliferation on 2,4-dicholorophenoxyacetic acid alone; (*g*) proliferated green friable regenerative callus on optimum medium combination; (*h*, *i*) shoot regeneration from callus; (*j*, *k*, *l*) multiplication of shoot clumps; *scale bars* = 10 mm.

Aldrich®, St. Louis, MO) for 3 min, then again thoroughly washed three to four times with autoclaved water.

Nutrient medium preparation Murashige and Skoog (MS; Murashige and Skoog 1962) basal salts augmented with 50 mg L^{-1} ascorbic acid, 25 mg L^{-1} each of arginine, adenine sulfate, and citric acid (Hi-Media®, Mumbai, India); and 3% (*w/v*) sucrose (Qualigens Fine Chemicals, Mumbai, India) was used as a basal medium for *in vitro* studies. Two different gelling agents, 0.8% (*w/v*) agar-agar (Bacteriological grade, Qualigens Fine Chemicals) and 0.16% (*w/v*) Phytagel™ (Sigma-Aldrich®), were compared. The pH was adjusted to 5.8 ± 0.02 using 1 N KOH or HCl (Loba Chemie, Mumbai, India) prior to autoclaving at 121°C and 1.1 kg cm^{-2} for 15 min.

Callus induction and proliferation The sterilized seeds were inoculated onto half-strength MS medium (macro and micro nutrients of MS basal reduced to half) with 0.2% (*w/v*) activated charcoal, supplemented with 500 μM gibberellic acid (GA_3; Sigma-Aldrich®) or 0.9 μM 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich®). After seed germination (Fig. 1*d*), whole seedling, or their hypocotyl, epicotyl, cotyledonary leaves, and roots, or leaf explants from mature plants (cut into 0.5 mm to 1 cm sections) were used for callus initiation. All these explants were placed onto MS basal medium supplemented with 2.27, 4.53, 9.06, or 13.59 μM 2,4-D. The cultures were incubated for 4 wk in a culture room at 26 ± 2°C with a 55–60% relative humidity (RH), and a light intensity of 40–50 μmol m^{-2} s^{-1} photon flux density (PFD) from white fluorescent tubes (Philips, India Ltd.; Mumbai, India) with a 12-h photoperiod.

For callus proliferation, the friable, competent, young calluses obtained from the hypocotyl part of young seedlings were transferred to medium containing different concentrations (Table 2) of 2,4-D, indole-3-acetic acid (IAA; Sigma-Aldrich®), or naphthaleneacetic acid (NAA; Sigma-Aldrich®), alone or in combination with 6-benzylaminopurine (BAP; Sigma-Aldrich®), and/or 6-furfuryl aminopurine (kinetin; Kin), and/or N6-(2-isopentenyl) adenine (2iP; Sigma-Aldrich®). The optimum proficient callus was sub-cultured three to four times onto fresh, identical medium at 4-wk intervals. Borosilicate (Borosil Glass Works Ltd., India) culture tubes (60 mL, 25 mm × 150 mm), Erlenmeyer conical flasks (100 mL; 64 × 105 mm; 150 mL, 72 × 124 mm and 250 mL; 85 × 145 mm), and screw-cap glass bottles (420 mL, 70 × 130 mm; Siddhivinayak Glass Concepts, Firozabad, India) were used to analyze the effect of culture vessel size and shape on callus proliferation and regeneration. About 15 mL of optimized callus proliferation medium was dispensed into 25 × 150-mm culture tubes, 20 to 50 mL dispensed into 100, 150, and 250-mL Erlenmeyer conical flasks and about 50 mL into each 420-mL screwed glass bottles.

Shoot regeneration from callus The friable callus was transferred onto MS basal medium solidified with agar-agar or Phytagel™ and fortified with different combinations of BAP, Kin, and/or TDZ with 2,4-D or NAA. Proliferating callus (approximately 200 to 400 mg per callus) was fragmented into 8 to 12 pieces and used for shoot regeneration. After regeneration of shoots, the clumps of callus were subcultured onto the same medium after an interval of 4 wk.

Effect of PGRs and medium composition on regenerated shoots *In vitro* regenerated shoots were harvested into clumps of 2 to 3 shoots and further cultured for analyzing the effects of culturing on the growth of shoots and the shape and size of leaves after 4 wk of culture on MS medium containing 2.22 μM BAP, 0.9 μM thidiazuron (TDZ, Sigma-Aldrich®), or 100 to 500 μM GA_3; full or half-strength MS basal salts; and agar-agar or Phytagel™.

***In vitro* rooting** Regenerated shoots were inoculated into quarter-, half-, or full-strength MS basal medium supplemented with 0.2% (*w/v*) activated charcoal (AC; Sigma-Aldrich®). Various concentrations of indole-3-butyric acid (IBA; Sigma-Aldrich®), 2,4-D, NAA, or IAA listed in Table 5, were added to the medium. For *in vitro* rooting, a pulse treatment method, in which shoot bases were treated with 490 μM IBA for 1 to 2 min and inoculated on MS medium containing 0.45 μM 2,4-D, 0.54 μM NAA, or 0.57 μM IAA or quarter-, half-, or full-strength MS basal medium was also used.

Transfer to *ex vitro* and hardening *In vitro* produced plantlets were taken out from culture vessels and transferred to glass bottles containing soil-rite (horticulture grade perlite with Irish peat moss mixture and exfoliated vermiculite (Kel Perlite, Bangalore, India)) and moistened with quarter strength of MS basal salt solution. The glass bottles were covered with polycarbonate caps and placed near the cooling pad end in a greenhouse where there was a high relative humidity (80–90%) and low temperature (28 ± 2°C). After 7 to 8 d, the caps of the bottles containing rooted plantlets were loosened and finally removed to acclimatize the *in vitro* grown plantlets to the external environment. Simultaneously, bottles were gradually shifted to the fan end of the greenhouse for further acclimatization where there was a low relative humidity (45–55%) and high temperature (36 ± 2°C). After 40 to 50 d, successfully hardened plants were transferred to polybags (Lodha Plastic Industries, Jodhpur, India) containing a mixture of soil-rite and field soil (1:1) and kept in greenhouse condition for 30 to 40 d and finally transferred to a nursery.

Leaf anatomy For anatomical comparisons, 25 to 30 leaves of approximately the same size of *M. nudicaulis* were taken from the 2nd tier of leaves from the base of 2 to 3-mo-old wild mature plants, from 4- to 5-wk-old aeroponically grown

Table 1. Effect of the type of explants and 2,4-dichlorophenoxyacetic acid (*2,4-D*) treatment on callus induction in *Mollugo nudicaulis*

Explants	2,4-D (μM)	Response			Callus induction %	Callus survival
		C	R	Sh		
Mature leaves	2.27	–	–	–	0.00	–
	4.53	C	R	–	52.47	Slimy callus, slow growing
	9.06	C	R	–	75.98	Brown; loose; rooty
	13.59	–	R	–	13.52	Direct root formation from callus
Hypocotyl	2.27	–	–	–	0.00	–
	4.53	C	R	–	87.04	Fast growing friable; rooting initiated
	9.06	C	R	–	97.78	Whitish creamy friable high rooty callus
	13.59	C	R	–	7.78	Vessels filled with enormous roots
Epicotyl	2.27	–	–	–	0.00	–
	4.53	C	–	–	18.70	Poor response
	9.06	C	R	–	42.12	White loose rooty callus
	13.59	C	R	–	4.37	Rooty grayish callus
Cotyledonary leaves	2.27	C	–	–	4.24	Very poor growth
	4.53	C	–	–	85.81	Very soft slimy callus
	9.06	C	R	–	91.59	Fast growing but watery and rooty
	13.59	C	R	–	11.07	Loose rooty
Root	2.27	–	–	–	0.00	–
	4.53	–	–	–	0.00	–
	9.06	C	–	–	3.46	Slow grown and soon dried
	13.59	C	–	–	2.22	Dried
Whole seedling	2.27	C	–	–	4.44	No further growth
	4.53	C	–	–	84.58	Friable, whitish, roots with new callus
	9.06	C	R	–	89.63	Whitish creamy friable, rapid growing
	13.59	C	R	–	13.70	Callus ended with roots

Observation based on qualitative character of friable or non-friable callus, root formation, and survivability after 4 wk of culture. *C* callus, *R* root, *Sh* shoot

plants, and from plants still in *in vitro* cultures. Transverse handmade cross sections were cut from fresh leaves and stained with 0.1% (*w/v*) safranin O or toluene blue (Sigma-Aldrich®) for 30 to 60 s and mounted on slides with distilled water. Photomicrographs were taken with a digital camera mounted on the CH20i binocular microscope (Olympus Corp., Tokyo, Japan) at 50× and 100× magnification.

Data recording and analysis All experiments were set up in a completely randomized block design (RBD) for single factor experiments (Compton and Mize 1999) and repeated three times with 15 replicates in each treatment. Percent response of callus, color and texture of callus, number of shoots, and number and shape of leaves were observed, and data was scored. Percent proliferation was measured by observing visual increase in volume of initial callus versus days. The data were analyzed statistically by analysis of variance test (ANOVA; Gomez and Gomez 1984) and differences among mean values were compared by Duncan's multiple range test (Duncan 1955) at $P < 0.05$ and alpha at 0.05 using Agricolae software package of R (Mendiburu 2016).

Results and discussion

Callus induction and proliferation Callus was induced from different types of juvenile explants as well as from mature leaf explants on various concentrations of 2,4-D (Table 1). The best responses were obtained with 9.06 μM 2,4-D applied to hypocotyl explants, cotyledonary leaves, and whole seedling, with 98%, 92%, and 90% of callus initiation, respectively (Table 1). However, hypocotyl explants produced the preferable white and friable callus (Fig. 1*e*), and cotyledonary leaves induced fast growing, but watery callus whereas callus obtained from whole seedling was friable whitish creamy and rapid growing (Table 1) but unsynchronized due to origin sites being mixed. In all explants, reduced response was observed at 13.59 μM 2,4-D and only roots were induced from cells of calluses in primary culture at this concentration (Table 1). For other plant species, the desired effect of 2,4-D for callus induction has been noted in previous reports (Chaâbani *et al.* 2015; Mohlakola *et al.* 2017).

For optimization of hypocotyl-induced callus proliferation, different plant growth regulators (PGRs), alone or in

Table 2. Effect of different plant growth regulators (*PGR*) on callus proliferation percentage, texture, and growth of callus originated from hypocotyl in *Mollugo nudicaulis*

PGR	Concentration (μM)	Proliferation percentage	Proliferation response	Proliferation time (d to response)	Growth, color, and texture
NAA	2.69	0.00 ± 0.00	–	0	–
	5.37	1.85 ± 2.62	*	14	No further growth
	8.06	21.99 ± 2.56	**	8	Slow, white, slimy, loose
	10.74	0.00 ± 0.00	**	7	Slow, white, slimy, loose
	13.43	46.06 ± 11.46	***	7	Fast, white, and fine granular
	16.11	48.61 ± 12.88	**	6	Fast growing, white, loose
	26.85	17.59 ± 12.49	Rooting	6	Rooty
2,4-D	2.27	0.00 ± 0.00	–	0	–
	4.53	10.42 ± 4.50	*	10	Slow and white
	6.8	17.35 ± 3.98	**	8	Friable, creamy white
	9.06	64.35 ± 5.82	***	7	Fast, creamy, friable,
	11.33	86.34 ± 5.79	****	5	Rapid growing, creamy, friable
	13.59	22.92 ± 7.41	**	5	Slower, whitish with roots
	22.65	6.08 ± 4.60	High rooting	5	Brown, rooty
IAA	0.57	0.00 ± 0.00	–	0	–
	2.86	0.00 ± 0.00	–	0	–
	5.71	0.00 ± 0.00	–	0	–
	11.42	0.00 ± 0.00	–	0	–
	17.13	4.63 ± 3.46	*	7	Very slow, compact, drying
2,4-D + BAP	11.33 + 0.89	84.26 ± 7.97	**	6	Fast, creamy, friable
	11.33 + 2.22	89.12 ± 5.68	***	4	Fast, fine granular, friable, pale creamy
	11.33 + 4.44	50.69 ± 11.33	*	7	Pale brown greenish; compact
NAA+ BAP	13.43 + 0.89	43.75 ± 8.84	**	10	Creamy, loose
	13.43 + 2.22	72.69 ± 8.51	***	7	Fast, green whitish, friable
	13.43 + 5.37	30.32 ± 13.99	**	10	Slow, whitish-green, compact
2,4-D + 2iP	11.33 + 0.98	64.58 ± 10.62	***	6	Fast, yellow-white, fine granular
	11.33 + 2.46	30.56 ± 3.93	**	14	Slower and whitish, compact
	11.33 + 4.92	23.84 ± 3.78	*	14	Slow whitish clump, compact
2,4-D + BAP+ Kin + 2iP	11.33 + 2.22 + 2.33 + 0.98	98.33 ± 2.36	****	4	Best growing, fastest growth, pale yellowish, granular, friable

MS basal medium (Murashige and Skoog 1962) + 3% (*w/v*) sugar + 0.8% (*w/v*) agar + PGR, singly or in combination, are shown

NAA naphthalene acetic acid, *2,4-D* 2,4-dichlorophenoxyacetic acid, *IAA* indole-3-acetic acid, *BAP* 6-benzylaminopurine, *2iP* N6-(2-isopentenyl) adenine, *d* days

Visual parameter ratings * = poor; ** = non friable; *** = good; **** = very good

combination, were added to the medium. When PGRs were used independently, maximum proliferation of callus (86%) was observed with 11.33 μM 2,4-D, with rapidly growing, creamy, and friable callus (Table 2). No callus proliferation response was observed on concentrations of IAA below 17.13 μM; however, among different NAA concentrations, optimum response (49%) was recorded with 16.11 μM NAA, proliferating a fast growing, white, and loose callus (Table 2).

Among different combinations of PGRs, the optimum response was observed with a combination of 11.33 μM 2,4-D, 2.22 μM BAP, 2.33 μM Kin, and 0.98 μM 2iP in terms of percent proliferation (98.33 ± 2.36%) with minimum of 4 d to respond (Table 2). The callus grown on medium containing both 2,4-D alone was cream colored, rooty, and friable (Fig. 1*f*) whereas the optimum proliferating callus obtained from the combined effect of 2,4-D, BAP, Kin, and 2iP was fast growing, pale-yellowish, and granular in appearance (Fig. 1*g*). Rathore *et al.* (2013) also found a positive effect of 2iP-inclusion with other PGRs on callus proliferation of in *Leptadenia reticulata* (Retz.) Wight & Arn.

Regarding the glass vessel effect, the best callus proliferation was observed in 250-mL Erlenmeyer flasks. Callus proliferated rapidly in comparison to other glassware used. The

Table 3. Effect of plant growth regulators (*PGR*) on *in vitro* shoot regeneration and growth (measured in number of shoots and leaves) of micro-plantlets in *Mollugo nudicaulis*

PGR	Concentration (μM)	Regeneration %	Number of shoots per culture vessel mean ± SD	Number of leaves per culture vessel mean ± SD
BAP + Kin + agar-agar	2.22 + 0	4.17	0.50 ± 0.55^{n}	1.00 ± 1.10^{m}
	0 + 2.33	6.17	0.50 ± 0.55^{n}	1.17 ± 0.98^{m}
	0.89 + 2.33	10.17	1.17 ± 0.75^{mn}	2.33 ± 1.37^{lm}
	2.22 + 0.98	27.54	2.50 ± 1.05^{lmn}	5.33 ± 2.66^{lm}
	2.22 + 2.33	39.58	4.00 ± 1.26^{kl}	8.50 ± 2.43^{lm}
	2.22 + 4.65	37.50	5.67 ± 2.34^{klm}	12.50 ± 6.35^{l}
	4.44 + 2.33	34.13	5.50 ± 1.22^{klm}	11.33 ± 3.93^{lm}
	4.44 + 4.65	39.58	5.00 ± 1.90^{klm}	12.33 ± 6.15^{l}
	4.44 + 9.8	41.67	4.50 ± 1.05^{jk}	10.17 ± 4.26^{lm}
	8.88 + 4.65	29.17	5.33 ± 2.80^{klm}	10.67 ± 4.41^{lm}
BAP + Kin + 2,4-D + agar-agar	2.22 + 2.33 + 0.91	68.75	12.17 ± 2.93^{ij}	27.17 ± 5.88^{k}
	2.22 + 4.65 + 0.91	75.00	13.00 ± 7.69^{ghi}	23.33 ± 9.75^{k}
	4.44 + 2.33 + 0.91	68.75	15.50 ± 1.97^{ghi}	45.33 ± 7.03^{hi}
	4.44 + 4.65 + 0.91	85.67	26.50 ± 7.23^{cd}	65.67 ± 6.71^{ef}
	4.44 + 4.65 + 2.69	62.50	19.83 ± 6.49^{ghi}	41.00 ± 5.48^{il}
	4.44 + 4.65 + 4.53	58.33	13.00 ± 2.53^{hi}	30.33 ± 6.41^{k}
	4.44 + 4.65 + 8.06	39.58	5.67 ± 1.03^{fgh}	11.33 ± 1.86^{lm}
BAP + Kin + NAA + agar-agar	2.22 + 2.33 + 1.07	68.75	13.50 ± 2.43^{hi}	30.67 ± 5.35^{k}
	2.22 + 4.65 + 1.07	56.25	11.67 ± 1.97^{ij}	24.33 ± 4.27^{k}
	4.44 + 2.33 + 1.07	62.50	15.50 ± 2.59^{hi}	33.17 ± 4.31^{jk}
	2.22 + 2.33 + 2.69	70.83	14.50 ± 3.45^{ghi}	32.60 ± 3.08^{jk}
	4.44 + 4.65 + 2.69	77.08	22.17 ± 1.72^{def}	46.33 ± 5.09^{hi}
	4.44 + 2.33 + 2.69	*100.0*	35.67 ± 4.37^{b}	57.67 ± 6.86^{lm}
	4.44 + 4.65 + 5.37	83.33	17.67 ± 1.75^{fgh}	33.17 ± 1.83^{jk}
	4.44 + 4.65 + 8.06	61.08	14.00 ± 1.67^{hi}	23.50 ± 3.78^{k}
	4.44 + 4.65 + 10.74	85.42	23.50 ± 3.45^{de}	56.83 ± 12.37^{l}
BAP + Kin + NAA + Phytagel™	4.44 + 4.65 + 2.69	*100.0*	34.00 ± 2.53^{b}	87.50 ± 7.06^{d}
	4.44 + 4.65 + 5.37	64.10	11.50 ± 2.59^{ij}	23.83 ± 4.79^{k}
	4.44 + 4.65 + 10.74	77.08	17.67 ± 5.89^{fgh}	51.50 ± 7.99^{gh}
BAP + Kin + NAA + TDZ + agar-agar	2.22 + 2.33 + 2.69 + 0.45	75.00	19.33 ± 6.89^{efg}	65.50 ± 9.20^{lm}
	2.22 + 2.33 + 2.69 + 0.91	87.50	29.00 ± 2.45^{c}	111.50 ± 6.60^{c}
	4.44 + 4.65 + 2.69 + 0.45	*100.00*	36.50 ± 1.05^{b}	125.10 ± 5.71^{b}
	4.44 + 4.65 + 2.69 + 0.91	*100.00*	13.00 ± 1.79^{hi}	26.50 ± 4.14^{k}
BAP + Kin + NAA + TDZ + Phytagel™	*4.44 + 4.65 + 2.69 + 0.91*	87.50	*41.17 ± 1.60*[a]	*141.50 ± 4.85*[a]
	4.44 + 4.65 + 2.69 + 1.36	81.25	23.33 ± 2.88^{de}	68.17 ± 8.73^{e}
	4.44 + 4.65 + 2.69 + 2.27	4.17	19.33 ± 3.61^{efg}	50.17 ± 11.74^{ghi}

Basal Murashige and Skoog (MS) medium (Murashige and Skoog 1962) + 3% (*w/v*) sucrose + additives. *Values* in a *column* followed by the *same letter* were not significantly different by Duncan's multiple rank comparison test (DMRT) at alpha = 0.05. Number of shoot and leaves were observed after 8 to 10 wk post-induction of regeneration, with sub-transfers every 4 wk maintaining medium and culture conditions

BAP 6-benzylaminopurine, *Kin* kinetin, *NAA* naphthalene acetic acid, *2,4-D* 2,4-dichlorophenoxyacetic acid, *TDZ* thidiazuron; Phytagel™ or agar-agar, solidifying agents. Italic value denotes maximum response

wider space of the conical flask might have favored the cell division due to better aeration and subsequent dilution of the ethylene effect. Glassware types affect the *in vitro* growth of many plants (Patel *et al.* 2016).

Shoot regeneration The optimization of the plant hormones in growth medium is necessary for long-term *in vitro* culturing and regeneration (Morini *et al.* 2000; Stobbe *et al.* 2002; Ziv and Altman 2003; Khan *et al.* 2009). For shoot regeneration

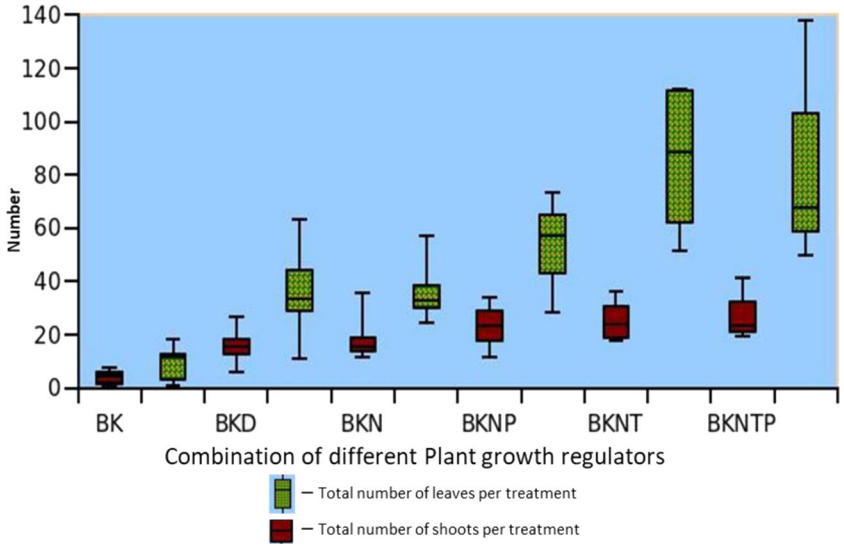

Figure 2. Comparative effects of different combinations of plant growth regulators on *Mollugo nudicaulis* Lam. shoot and leaf production. *Dark box*, total number of shoots per treatment. *Light box*, total number of leaves per treatment. Medium was solidified with agar-agar unless otherwise indicated. *BK*, 6-benzylaminopurine (BAP) + kinetin (Kin); *BKD*, BAP + Kin + 2,4-dicholorophenoxyacetic acid (2,4-D); *BKN*, BAP + Kin + naphthaleneacetic acid (NAA); *BKNP*, BAP + Kin + NAA + Phytagel™ (solidifying agent); *BKNT*, BAP + Kin + NAA + thidiazuron (TDZ); and *BKNTP*, BAP + Kin + NAA + TDZ + Phytagel™. *Whiskers as box bar* = Lowest and highest observed value, *center line in box* = median.

from proliferating callus, different PGR combinations (Table 3) were used. Maximum regeneration percent response of 100% was observed on MS basal medium supplemented with 4.44 μM BAP, 4.65 μM Kin, and 2.69 μM NAA and solidified with Phytagel™. The maximum response in terms of the number of shoots (36.50 ± 1.05) was observed on a regeneration medium containing 4.44 μM BAP, 4.65 μM Kin, 2.69 μM NAA, and 0.91 μM TDZ (Table 3; Fig. 1*h*). Thidiazuron, an active cytokinin-like phytohormone, has been reported in many plants to mimic the functions of both auxin and cytokinin, when added in minute concentrations to the medium (Huetteman and Preece 1993; Morini *et al.* 2000; Sharma *et al.* 2017). The exogenous levels of plant hormones in culture medium have been observed to be a significant factor for induction of organogenesis in friable callus (Stobbe *et al.* 2002).

Table 4. Response of regenerated *Mollugo nudicaulis* plantlets on different growth medium compositions

Treatment combinations	Effect on *in vitro* plantlets
HF	Leaves became curly and brown, no further growth
MS (macro half)	Shoots dried
2.22 μM BAP	Two- to three-fold increased number of small axillary buds at base, slow growth, and thin leaves
100–500 μM GA_3	*In vitro* flowering and leaf elongation
0.9 μM TDZ + agar-agar	Shoots survived, no further increase in growth
0.9 μM TDZ + Phytagel™	Adventitious shoot formation from leaves, but leaves vitrified
Phytagel™ + BKNT (optimized in regeneration)	Increased width and length of old leaves, number of new young leaves, and shoots, and *in vitro* flowering induced. Delayed *in vitro* flowering and seed setting
2.22 μM BAP + 100 μM GA_3	Leaf width increased, showing typical shape of the plant and *in vitro* flowering induced

HF hormone free, *MS macro half*, Murashige and Skoog medium (Murashige and Skoog 1962) with half concentrations of macronutrients of MS basal medium, *BAP* 6-benzylaminopurine, *GA3* gibberellic acid, *TDZ* thidiazuron, *BKNT* regeneration optimized concentrations of 4.44 μM BAP + 4.65 μM Kinetin + 2.69 μM naphthalene acetic acid (NAA) + 0.91 μM TDZ. Observation based on healthy green leaves, *in vitro* budding, seed setting, and new activation of shoot bud–break

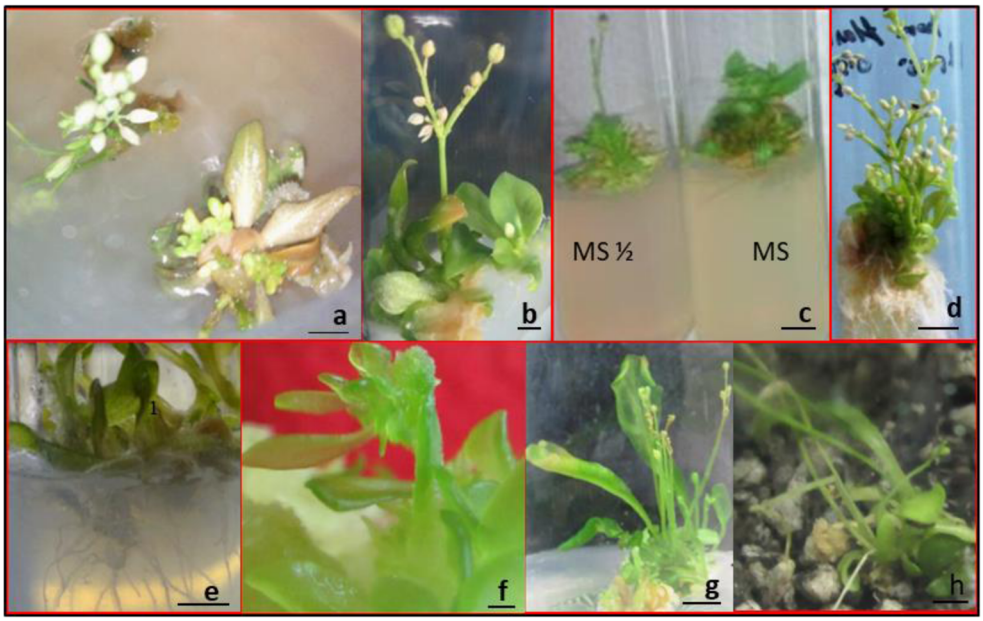

Figure3. Depiction of responses of *in vitro* raised *Mollugo nudicaulis* Lam. plantlets. (*a*) Effects of gibberellic acid (GA_3): adventitious bud activation and *in vitro* flowering. (*b*) Combined effect of 6-benzylaminopurine (BAP) and GA_3: typical leaf shape and thickness, *in vitro* flowering, and seed formation. (*c*) Effect of Murashige and Skoog (MS; Murashige and Skoog 1962) strength medium on shoot growth. (*d*) Rooted shoots on MS medium containing 0.45 μM 2,4-dicholorophenoxyacetic acid after indole-3-butyric acid (IBA) pulse treatment. (*e*) Rooting on half strength MS with activated charcoal after IBA pulse treatment. (*f*) Direct shoot formation on 0.91 μM thidiazuron and Phytagel™. (*g*) Growth on MS supplemented with 2.22 μM BAP. (*h*) Plantlets under hardening process. *Scale bars* = 5 mm.

Use of 0.16% (*w/v*) Phytagel™ instead of agar-agar in the above combination enhanced the response in terms of the number of shoots (41.17 ± 1.6), as well as number of leaves (141.50 ± 4.85) per culture vessel, with an overall shoot regeneration percentage of 87.50% (Table 3; Fig. 1*i*). The superiority of Phytagel™ over other gelling agents for most plant culture systems may be due to its consistent texture and high purity (Huang *et al.* 1995).

Total biomass production in terms of number of shoots and leaves per treatment was compared for different PGR combinations (Fig. 2) and demonstrated that including a low concentration of TDZ in the medium increased the total biomass production by encouraging axillary bud formation, which is consistent with the finding that TDZ prompts axillary proliferation, callus formation, shoot organogenesis, and somatic embryogenesis in many tissue-cultured species (Huetteman and Preece 1993; Morini *et al.* 2000; Yancheva *et al.* 2003; Sharma *et al.* 2017). The average of total biomass production in each category of treatments was increased two- to three-fold when Phytagel™ solidified medium supplemented with TDZ with other PGRs were used. The superior effect of Phytagel™ over agar-agar on *in vitro* shoot multiplication and growth has been reported in *Chlorophytum borivilianum* (Kumar et al. 2010).

Effect of the PGRs and medium composition on regenerated shoots Regenerated shoots were cultured on MS medium containing different PGRs, and their growth responses were noted (Table 4). The number of shoot buds were two- to three-fold higher upon the addition of 2.22 μM BAP to the medium (Table 4). *In vitro* flowering was observed when GA_3 was added to the medium (Fig. 3*a*, *b*). Huh *et al.* (2017) reported similar effects of GA_3 and BAP in *Passiflora edulis* Sims. When cultured on 2.22 μM BAP, shoots produced a higher number of axillary buds, whereas on 0.9 μM TDZ-containing medium, shoots were hyper hydrated/vitrified and produced adventitious shoots (Table 4; Fig. 3*f*, *g*).

In order to analyze the effect of MS medium strength, the control shoots were cultured on MS basal and half-strength MS medium without any PGR, and they started drying after 1 wk of culture and showed no significant growth (Table 4; Fig. 3*c*). Addition of BAP, GA_3, or TDZ to medium increased the survivability and growth of the *in vitro* raised plantlets.

***In vitro* rooting** Rooting was induced in the regenerated plantlets using medium containing various combinations of auxins with 0.2% (*w/v*) AC. *In vitro* rooting was only obtained after a pulse treatment of 490 μM IBA was applied to shoot basal sections for 2 to 3 min. Two weeks after the IBA treatment, root development could be observed (Fig. 3*d*, *e*). The effectiveness of the synthetic auxin IBA for *ex* and *in vitro* rooting might be due to its preferential transportation in the acropetal direction by plant cells and its ability to activate the

Table 5. Effect of medium strength and different plant growth regulators on *in vitro* rooting of *Mollugo nudicaulis* with or without IBA pulse treatment

Treatment	Concentration (μM)	Remarks
MS ¼	HF	No rooting
MS ½	HF	No rooting
MS basal	HF	No rooting
IBA	2.45	Callogenesis, no rooting
	4.90	Callogenesis, no rooting
	7.35	Callogenesis, no rooting
	9.8	Callogenesis, no rooting
2,4-D	0.45	Callogenesis, no rooting
	2.69	Callogenesis, no rooting
	4.53	Callogenesis, no rooting
	6.8	Callogenesis, no rooting
	9.06	Callogenesis, no rooting
NAA	0.54	Callogenesis, no rooting
	2.69	Callogenesis, no rooting
	5.37	Callogenesis, no rooting
	8.06	Callogenesis, no rooting
	10.74	Callogenesis, no rooting
IAA	0.57	Callogenesis, no rooting
	2.87	Callogenesis, no rooting
	5.71	Callogenesis, no rooting
	8.57	Callogenesis, no rooting
	11.42	Callogenesis, no rooting
MS ¼ (pulse treated)	HF	Poor *in vitro* rooting
MS ½ (pulse treated)	HF	Healthy and elongated roots no callusing
MS basal	HF	Moderate rooting
2,4-D (pulse treated)	0.45	Proliferated and Elongated Rooting with Callusing at base of shoot
NAA (pulse treated)	0.54	Moderate *in vitro* rooting
IAA (pulse treated)	0.57	Poor *in vitro* rooting

MS Murashige and Skoog basal medium (Murashige and Skoog 1962), *IBA* indole-3-butyric acid, *2,4-D* 2,4-dichlorophenoxyacetic acid, *NAA* naphthalene acetic acid, *IAA* indole-3-acetic acid. *IBA* concentration for pulse treatment = 490 μM

successively interacting genes necessary for rooting (Ludwig-Müller 2000; Rufai *et al.* 2016; Sharma *et al.* 2017). However, the best response was observed on both lower concentrations of 2,4-D and half-strength MS medium in terms of number and size of roots (Table 5). When 0.45 μM 2,4-D was incorporated into MS basal medium, *in vitro* rooting was established within 2 wk, whereas on pulse treated half-strength hormone free (HF) MS medium roots were induced after more than 3 wk. *In vitro* rooted plantlets were transferred to the greenhouse with successful hardening and the plantlets completed their life cycle after seed formation within 1 to 2 mo (based on observation); this was probably due to the plant's annual habit (Fig. 3*h*).

In the present study of this C_3–C_4 intermediate species, it was observed that once *in vitro* shoots regenerated and formed plantlets in *M. nudicaulis*, they utilized the available resources as fast as they could as evidenced by rapid production of in *vitro* flowering and seed setting in 3 to 4 wk in *in vitro* conditions, compared to the *ex vitro* grown plants of *M. nudicaulis* that flowered and set seed in 4 to 6 wk after emergence of seedlings.

Leaf anatomy In physiological adaptations, leaf anatomy is a key indicator for the plant's photosynthetic type and its capability to thrive in a specific environment (Terashima *et al.* 2011; Tholen *et al.* 2012). Determination of gross leaf anatomy under the light microscope allowed the identification of the anatomical adaptations at an initial level regarding photosynthetic tissue differentiation and organization in leaves.

Under the microscope, the leaf anatomy of natural habitat plants showed more compact tissue organization, compared to aeroponically grown and *in vitro* grown leaves (Fig 4). In naturally grown leaves, this study

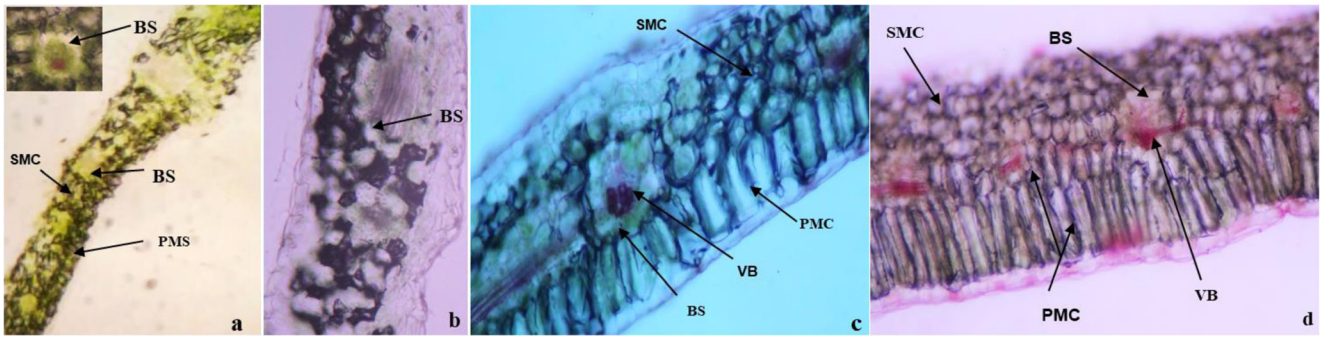

Figure 4. Comparison of leaf anatomy of free hand transverse sections of (*a, b*) *in vitro,* (*c*) aeroponically grown, and *(d*) mature *ex vitro* leaf of *Mollugo nudicaulis* Lam. observed under light microscope at 50× (*a*) and 100× magnification (*inset*, *b*, *c and d*). *Inset* shows enlarge view of a vascular bundle of *in vitro* leaf section. (*a*) *In vitro* leaf at 50× showed loosely arranged mesophyll cells with single layer of palisade (*PMS*), but bundle sheath (*BS*; *arrow*, *inset*), and (*b*) with centripetal arranged chloroplast was present around a vascular bundle, whereas (*c*) the aeroponic leaf section showed distinct single layer of palisade mesophyll cells (*PMC*; *arrow*), compact spongy mesophyll cells (*SMC*; *arrow*), and clear bundle sheath (*BS*; *arrow*) around vascular bundle (*VB*; *arrow*) with centripetal chloroplasts; by comparison, (*d*) the natural habitat leaf was dark green in color and showed two layers of palisade cells (*PMC*; *branched arrow*), compact spongy mesophyll cells (*SMC*; *arrow*), and bundle sheath (*BS*; *arrow*) with chloroplasts compactly surrounded by mesophyll cells. *50*× = 5 × 10 and *100*× = 10 × 10 (power of objective × ocular lens).

revealed the distinct differentiation of mesophyll tissues into two-layered palisade cells and compact spongy cells with abundant inclusion of chloroplasts (leaves were dark green; Fig. 4*d*), as generally found in C_3 plants under direct sunlight (Stata *et al.* 2014). Furthermore, a clear Kranz anatomy (concentrically arranged bundle sheath cells and mesophyll cells around a vascular bundle) indicative of C_4 plants, as reported by Kennedy *et al.* (1980) in *Mollugo* spp., was less evident in these leaves. In contrast, the transverse sections of aeroponically grown leaves (Fig. 4*c*) showed the presence of single-layered palisade cells, loose spongy mesophyll cells, and the presence of a distinct Kranz morphology with a single layer of bundle sheath cells, including centripetally arranged chloroplasts (green color accumulated toward center) around the vascular bundle. These leaves were a light-green color, likely due to growing in a controlled greenhouse environment.

An *in vitro* environment consists of constant temperature, low photosynthetic photon flux density, large diurnal fluctuation in CO_2 concentration, high relative humidity level, high concentrations of sugar, salts, PGRs, and nutrients in the medium, and the absence of microorganisms (Xiao *et al.* 2011; Kaur 2015), and thus often leads to low rates of transpiration, photosynthesis, and water, nutrient, and CO_2 uptake, all of which can result in poor growth and physiological adaptations in the *in vitro*-grown plant (Xiao e*t al.* 2011). In the present study, microscopic examination of transverse handmade sections of *M. nudicaulis* from *in vitro* conditions (Fig. 4*a*, *b*) revealed similar findings as less compact and less differentiate mesophyll cells (present as uncleared spongy mesophyll and single layer of palisade cells), an underdeveloped cuticle, and overall, the leaves were a very delicate and lighter green, compared to other two leaf types observed. In previous studies, Hazarika (2006) reviewed that the poor development of photosynthetic apparatus in *in vitro* leaves may be due to the continuous exogenous supply of high sucrose and salt in media and poor light conditions. However, in *M. nudicaulis*, the presence of bundle sheath cells with chloroplast arranged centripetally around the vascular bundles (Kranz system) was observed in the *in vitro*-derived leaves (Fig. 4*a*, *b*) which is indicative of C_3-C_4 intermediacy.

The presence of all types of photosynthetic tissues (palisade and spongy mesophyll cells, and Kranz system with dense chloroplasts in bundle sheath cells) in a leaf is considered as characteristic for C_3–C_4 plant intermediates at the anatomical level (Kennedy *et al.* 1980). The bundle sheath cells typically demark the inner compartment of cells where the enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is localized and high levels of CO_2 are found (Sage *et al.* 2014; Stata *et al.* 2014). Thus, in this comparison, leaves from both the aeroponically and *in vitro*-grown plants showed characteristics closer to C_4 species compared to *ex vitro*-grown *M. nudicaulis*. However, an empirical experiment will be needed to observe an effect on physiological adaptations in aeroponically and *in vitro*-grown plants compared to naturally grown C_3 or C_4 plants. This present study is first to report the comparison of *M. nudicaulis in vitro* and *ex vitro* (both, native habitat and aeroponic) derived leaves as demonstrating anatomical evidence for this C_3–C_4 intermediate species.

Conclusions

For the first time, the *in vitro* growth responses and simple comparative leaf anatomy in the C_3–C_4 intermediate medicinal plant *M. nudicaulis* were studied. From the results, it can be concluded that the hypocotyl was best for callus induction

in *M. nudicaulis* and that callus induction required a high concentration of auxin. At the leaf anatomy level, *in vitro*- and aeroponically grown *M. nudicaulis* plants showed increased intermediate characteristics, compared to plants grown in their natural habitat. This tissue culture protocol could be used for year-round availability of the plant, as well as allowing for comparative analysis of the functional anatomy of the aeroponic- and *in vitro*-grown plants of other C_3 and C_4 species.

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