This is a post-peer-review, pre-copyedit version of an article published in Mycorrhiza. The final authenticated version is available online at: https://doi.org/10.1007/s00572-018-0867-3

Mycelium of *Terfezia claveryi* as inoculum source to produce desert truffle mycorrhizal plants

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Abstract

Terfezia claveryi Chatin was the first desert truffle species to be cultivated, the mycorrhizal plants being successfully produced by using both desert truffle spores and mycelia. However, it is more advisable to use mycelium than spores whenever possible and profitable. Given the low yields of mycelia obtained using traditional culture methods of this truffle, the medium composition was modified in an attempt to determine its nutritional requirements. For this, an assay involving response surface methodology was performed using Box-Behnken design to find the optimal parameters for the high production of mycelial biomass. The best results were obtained with glucose as carbon source, buffering the pH at 5 during culture, adding a pool of vitamins and adjusting the optimal concentrations of carbon and nitrogen sources of the MMN medium. Biomass production increased from 0.3 to 3 g L^{-1} dry weight and productivity increased from 10.7 to 95.8 mg L^{-1} d^{-1} dry weight. The produced mycelium was able to colonize *Helianthemum* roots efficiently, providing more than 50 % ectomycorrhizal colonization.

Keywords mycelial biomass; Box-Behnken design; response surface methodology; desert truffle; *Terfezia claveryi*

29

30 **Introduction**

31 The term ‘desert truffles’ comprises species of different hypogeous Ascomycetes genera, such as
32 *Terfezia*, *Picoa*, *Tirmania*, *Balsamia*, *Delastria*, *Phaeangium* and some *Tuber* species, which are typical
33 of countries or territories with arid and semiarid conditions. Among desert truffles, several genera have an
34 excellent record as edible fungi and, two of them are of considerable economic importance: *Terfezia and*
35 *Tirmania* (Morte et al. 2009). *Terfezia claveryi* Chatin was the first desert truffle species to be cultivated
36 and numerous desert truffle plantations have been established in Spain in the last ten years, the first
37 ascocarps normally appearing two years after plantation (Morte et al. 2017).

38 In natural ecosystems, *T. claveryi* establishes mycorrhizal symbiosis with numerous species of
39 the genus *Helianthemum*. Nowadays, the increasing demand for this novel crop, not only in Spain but
40 also in other countries, has prompted the research for new strategies to help pass from experimental scale
41 to large-scale cultivation (Morte et al. 2012, 2017; Navarro-Ródenas et al. 2016). Mycorrhizal plants have
42 been successfully produced by using both desert truffle spores and mycelia (Morte et al. 2008). Some
43 advantages of using spore-based inoculations are that inoculum is easy to prepare and less time
44 consuming, relativey cheap, and it does not require specialized equipment or training. However, the
45 problem with using spore inoculation is that spores can carry pests, pathogens and other mycorrhizal
46 fungi which can contaminate the mycorrhizal plants (Iotti et al. 2016). Therefore, it is more advisable to
47 use mycelium than spores whenever possible.

48 *T. claveryi* presents very slow mycelium growth *in vitro* and most of the strains isolated were not
49 able to grow after subculturing (Navarro-Ródenas et al. 2011). Soil mycelium of *T. claveryi* is
50 intermediate between “contact exploration type” and “short-distance exploration type”, which is
51 indicative of its slow growth (Honrubia et al. 2014). Although, it has been reported that the mycelium of
52 *T. claveryi* grows better under a moderate water stress (Navarro-Ródenas et al. 2011) and in the presence
53 of β-cyclodextrin in the culture medium (López-Nicolás et al. 2013), these improvements are not
54 sufficient to obtain enough mycelial biomass for use as inoculum in plant nursery production on a semi-
55 industrial scale. The slow or erratic growth of fungi could be improved by optimizing the conditions
56 and/or composition of the culture medium in bioreactors, as has been observed in other ectomycorrhizal
57 (ECM) fungi such us *Pisolithus tinctorius* (Pradella et al. 1991), *Pisolithus microcarpus* (Rossi et al.

2002), *Rhizopogon nigrescens* (Liu et al. 2008), *Tuber melanosporum* (Liu et al. 2009), *Coriolus versicolor* (Wang et al. 2012) and *Lactarius deliciosus* and *Suillus mediterraneensis* (Carrillo et al. 2004), among others.

Most ECM fungi, known as pioneer fungi, can be grown in Modified Melin Norkans (MMN, Marx 1969) medium, but some genera require a richer medium for optimal growth. This is the case for some species of *Amanita*, *Lactarius* and *Russula*, which grow better when Biotin-Aneurin-Folic Acid (BAF) (Moser 1960) is used as culture medium. The MMN medium contains 3 g L^{-1} of malt extract but was eliminated in the case of *T. claveryi* culture because it did not improve growth (Morte and Honrubia 1994).

A previous study of mycelial inoculum production of the desert truffle *Terfezia olbiensis* (Morte et al. 2004) in bioreactor pointed to a lag phase of 15 days and 1.16 g L^{-1} in dry weight of biomass after 29 days of fermentation in MMN medium. Although there are numerous studies on the aerobic liquid culture of ECM fungi in fermentation tanks, information on the optimal methodology and yield is scarce. Box-Behnken experimental design, through response surface methodology (RSM), has been increasingly used to optimize microorganism fermentation processes (Mao et al. 2005; Liu and Wang 2007; Wei et al. 2014; Kumar and Mishra 2017). This methodology allows multiple variables and the interactions among them to be tested with the added advantage of reducing the experimental trials (Ferreira et al. 2007).

The objective of this work was to design a culture medium where *T. claveryi* can grow quickly and efficiently, testing the effect of macronutrients, micronutrients and vitamins on the mycelial growth *in vitro*. After that, carbon and nitrogen concentrations and pH were optimized for the efficient production of biomass and the culture medium was readjusted to enhance this growth. Finally, the ability of the obtained mycelium to form mycorrhizas in *Helianthemum almeriense* plants was tested.

Materials and methods

An outline of the different experiments detailed below is shown in Fig. S1.

Fungal material and preculture

T. claveryi mycelium, strain T7, isolated from ascocarps collected in Zarzadilla de Totana (Lorca, Murcia, Spain, N 37º 52’ 14.308’’, O 1º 42’ 6.71’’), with alkaline soil (pH 8.0), under plants of *Helianthemum almeriense* Pau (Fig. 1a), was maintained in solid Petri dishes (Fig. 1b) in MMN medium

without malt extract, pH 7.0, 24ºC and in darkness (stock cultures). To prepare the culture media, all carbon sources and vitamins were sterilized by filtering using 0.22 µm Millipore filters and added to the previously autoclaved medium once cooled. Plugs of mycelium-agar were used to inoculate the liquid medium cultures to generate precultures. Previous to the assays in the different liquid media, precultures in 200 mL flasks were performed to activate mycelium growth and obtain enough mycelium biomass to carry out the different assays (Fig. 1c).

Determination of dry weight of the mycelial biomass produced, its residual volume and residual glucose and ammonium (NH_4^+)

Both initial and final biomass in 200 mL flasks was quantified as mycelial dry weight. The mycelium was filtered, washed with distilled water and dried in an oven at 60ºC for 72 hours until the weight was constant. In order to calculate the biomass produced, the following equation was used:

$$B = \frac{(B_f - B_i)}{Vr} \quad (1),$$

where, B corresponds to the total biomass concentration (g L^{-1}), B_f is the final biomass (g), B_i is the initial biomass (g) and Vr is the residual volume of the culture medium (L).

Residual glucose was measured by spectrophotometry using the glucose oxidase method (Trinder 1969; Lott and Turner 1975), with the QCA® kit. The reaction product was measured at 505 nm and the resulting data were compared with those of the glucose standard. Residual ammonium (NH_4^+) was determined by a colorimetric kit (JBL®NH4 TEST). The data were measured at 690 nm and the concentrations were calculated from a standard curve previously obtained using $(NH_4)_2HPO_4$ from 0.1 to 5.0 mg L^{-1}.

Bioassay 1: Macronutrients and carbon source screening

Strain T7 was grown in glucose, sucrose and mannitol as carbon source. For this screening, a factorial test of two factors, carbon source and percentage (5% and 10%) of initial inoculum size (v/v), with five replicates per treatment in MMN medium (Table 1), at pH 7.0, was designed. Flasks containing 5 and 10 mL of mycelial preculture (5 and 10% of initial inoculum size, respectively) in 100 mL of culture medium were incubated for 30 days at 24ºC, with stirring of 100 rpm, in darkness.

Another treatment consisting of 3-fold increasing concentrations of the MMN macronutrients (Table 1), called MMN 3x Macronutrients, under the same culture conditions, was studied.

Bioassay 2: Micronutrients and vitamins screening

A pool of selected micronutrients and vitamins from other common culture media –minimal medium, M (Bécard and Fortin 1988) and BAF medium– were added to the MMN medium. Three different treatments, with twenty replicates of each, were designed: MMN as a control group, MMN plus micronutrients and MMN plus vitamins and glycine (Table 1). Plugs of 5 mm were taken from the colony edge of *T. claveryi* stock plates showing active growth and grown on cellophane agar plates at 24ºC in darkness for 45 days. The obtained mycelial biomass was measured by weighting the colonies growing over cellophane sheets, after they were dried in an oven at 60ºC for 72 hours until the weight was constant.

The study of the growth kinetic in liquid culture was carried out in two mycelium cultures involving MMN and MMN plus vitamins (a total of 40 flasks each containing 50 mL) and incubated at 24ºC in darkness, at pH 7.0, during six weeks. Using the 10% (v/v) initial inoculum (from a 7-day-preculture), 6 random flasks were harvested each week and the mycelial biomass was measured and calculated by Eq. 1. Then, a growth profile of T7 strain was made comparing both culture media.

Bioassay 3: Optimization by means of the Box-Behnken experimental design

In an attempt to improve the process, the pH, nitrogen source and carbon source concentrations were selected for further optimization studies using a Box-Behnken design (Box and Behnken 1960). This method is an independent quadratic model where the combinations of treatments are at the midpoints of edges of the process space and at the centre point (Natrella 2010). The lowest and the highest levels of the variables were: pH (x_1), 5 and 7; $(NH_4)_2HPO4$ (x_2), 0.25 and 0.75 g L^{-1}; and glucose (x_3), 5 and 15 g L^{-1} (Table S1).

The experiment consisted of 15 trials with 5 replicates and 3 levels for each factor (three in this case), in which combinations of independent variables were studied to estimate the error. Flasks containing 100 mL of MMN-Vitamins medium with the above-mentioned changes were incubated at 24ºC in darknes on a rotatory shaker at 100 rpm. To keep the pH buffered for each treatment (5, 6 and 7),

MES hydrate (2-(N-Morpholino) ethanesulfonic acid hydrate) was added at 10 g L^{-1} (0.05M). After 32 days, flasks were harvested and the biomass produced (Eq. 1), glucose and residual NH_4^+ were measured.

The optimal conditions thus obtained were tested in a 5 L stirred tank bioreactor (Applikon®Biotechnology) culture to verify the model (Fig. 1d). The initial inoculum was cultivated for 15 days before the mycelium was transferred to the bioreactor. MMN-Optimized medium was used (Table 1) and the following parameters were monitored: temperature 24ºC, pH 5.2 (adding 0.5 N NaOH if there is acidification of the medium due to fungal growth), constant stirring at 100 rpm, air-flow 0.5 L/min and dissolved oxygen (% DO) in excess of 60 % (Morte et al. 2004). The final cultivation volume was 3.5 L and the starting inoculum consisted of 10 % mycelium [v/v] precultured (3.15 L plus 350 mL of initial inoculum). Finally, total produced mycelial biomasses were calculated according to Eq. 1.

Mycorrhizal symbiosis ability in *Helianthemum almeriense*

The mycelium produced in the fermentation assay was tested to confirm its ability for producing mycorrhizal plants. After harvesting the mycelium from the bioreactor, it was filtered, washed and homogeneized, with a sterile blender, in the same volume of sterile water before inoculation. Then, for *in vitro* checking, a total of 24 vermiculite tubes with *H. almeriense* plantlets watered with MH medium (Morte and Honrubia 1994) were inoculated with 2 mL per tube of that mycelial suspension. In addition, another 24 vermiculite tubes were prepared without mycelial inoculum as control samples. The culture conditions were those proposed by Morte and Honrubia (1994, 1997). For non-aseptic tests conditions, ten-week-old seedlings were transplanted and inoculated with approximately 3 mL of mycelial suspension in each pot (140 pots) containing soil/black peat/perlite [1:1:1 (v/v)] and grown in greenhouse as detailed in Navarro-Ródenas et al. (2016).

Two-month-old plants were analysed to measure the mycorrhizal colonization on stained root samples as previously described (Gutiérrez et al. 2003) and the percentage of mycorrhization was visually estimated (Giovannetti and Mosse 1980) under an optical microscope.

Growth test of *T. claveryi* strains on MMN-Optimized medium

MMN-Optimized medium was tested with another four *T. claveryi* strains (T1, T2, T5 and T9) with the MMN medium as a control. Plugs of 5 mm were taken from the colony edge of *T. claveryi* stock plates and eight replicates of each strain were grown on cellophane agar plates at 24ºC in darkness for 8

weeks. After this period, colony areas were measured with ImageJ program (Scheiner et al. 2012) and the mycelial biomass was measured by weighting the colonies growing over cellophane sheets, after they were dried in an oven at 60ºC for 72 hours until the weight was constant.

Genetic analysis by polymerase chain reaction

A DNA analysis was carried out by PCR (polymerase chain reaction) to confirm and check that the fungal biomass belonged to *T. claveryi*, and was free of contaminants. The DNA was extracted by the C-TAB method (Chang et al. 1993) and amplified using fungal specific primers ITS1F and ITS4 according to Bordallo et al. (2013). The amplified fragments were sequenced and compared in the Genbank database (NCBI). The results confirmed that the mycelium was *Terfezia claveryi* and was free of contaminants throughout the experiment.

Statistical analysis

The assumptions of normality and homocedasticity (homogeneity of variance) were corroborated. Data were subjected to ANOVA I and ANOVA II in a factorial design, according to Tukey's test or Dunnett's test. Statistical analysis was carried out using the software package SPSS (version 15). Additionally, for the BBD assay, a mathematical model to describe the effects between the independent variables was developed using the following second-order equation:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1=j}^{3} \beta_{ij} x_i x_j + \xi \quad (i = 1,3; j = 1,3, i \neq j) \quad (2),$$

where y is the predicted response variable; β_0, β_i, β_{ii}, and β_{ij} are constant regression coeficients of the model; x_i, and x_j represent the independent variables in the form of coded values, and ξ is the random error (effects not explained by the model). Then, an ANOVA test was performed on the results to evaluate the statistical significance of the model and using Modde 5.0 Umetrics AB statistical package and software for multiple lineal regression analysis and the graphical optimization (response surface methodology, RSM).

Results and discussion

Screening of different nutrients and the size of initial inoculum

The effect of increased nutrients that were thought to either improve or decrease mycelial growth was checked. Firstly, the highest biomass production was obtained using both glucose as carbon source

194 and 10 % of the initial inoculum size (Fig. 2a, Table S2). This treatment provided significantly better
195 results than the other treatments. However, there were no differences between the carbon sources when 5
196 % of initial inoculum size was applied (Table S2). This means that sucrose and mannitol but not glucose
197 could be growth limiting as carbon sources, when working with volumes above 10% [v/v] of starting
198 inoculum. Taking into account the type of nutrition and growth of fungi, it is important to know the ratio
199 between inoculum size and volume of the medium (Cochrane 1958; Jennings 1995). Concentrations of
200 different metabolites might play an important role in the growth of these symbiotic fungi, whose growth
201 *in vitro* is slow and difficult in pure culture. Moreover, the maintenance of a successful long-term
202 relationship seems strongly regulated by resource allocation between symbiotic partners, suggesting that
203 nutrients themselves may serve as signals (Garcia et al. 2015). Wang et al. (2012) obtained the highest
204 mycelial production for *Coriolus versicolor* with malt extract as a source of carbon and 8% of initial
205 inoculum size, which normally range between 3% and 10% of the culture (Stanbury et al. 2013). Our
206 results showed that the T7 strain grew better with higher initial inoculum (Fig. 2a), and that the biomass
207 produced was double (0.61 g L^{-1}) than that produced with half the inoculum (0.33 g L^{-1}) when glucose is
208 used as carbon source (Table S2).

209 The results showed that the size of initial inoculum (Fig. 2a), but not the addition of
210 macronutrients to the culture medium affected the mycelial biomass production rate (Fig. 2b). About 0.3
211 g L^{-1} and 0.6 g L^{-1} were obtained in both media, using 5% and 10 % of the initial inocula, respectively
212 (Fig. 2b). Therefore, macronutrients of the MMN medium were not limiting the growth for the T7 strain.
213 Then, the MMN medium was used for the following experiments in a further attempt to identify the
214 nutritional requirements.

215 In the second bioassay, the effects of a pool of micronutrients and vitamins added to MMN
216 culture medium were analyzed. The only significant differences observed were in MMN medium with
217 added vitamins (Fig. 3), according to Dunnett's test ($p<0.01$). The micronutrients did not increase the
218 mycelial biomass significantly. It is known that fungi require several vitamins and growth factors to grow
219 and spread (Cochrane 1958; Jennings 1995). These requirements may depend on the fungal strain or
220 species and sometimes on the culture conditions.

221 When the *T. claveryi* strain T7 growth profile was characterized, different growth phases were
222 obtained (Fig. 4). The exponential phase of the fungus grown in MMN plus vitamins was seven days

shorter than that in the control medium without vitamins, and the biomass obtained was higher (3 g L^{-1} vs. 1.3 g L^{-1}) (Fig. 4). Also, we could observe that a faster growth rate (slope of curve in exponential phase) was obtained when MMN plus vitamins was used.

The residual glucose was lower in the medium with added vitamins (3 g L^{-1}) than the MMN without vitamins (5 g L^{-1}), whereas the ammonium was almost totally consumed in both media (data not shown). Since ECM fungi need more carbon source to efficiently assimilate nitrogen, some authors have reported a reduced ECM mycelium growth with high concentrations of nitrogen source under a limiting carbon source (Garcia et al. 2015). In *T. claveryi*, the nitrogen source could be limiting growth because it was completely consumed, despite the excess glucose remaining in the medium at the end of the culture when the initial concentration of nitrogen source was 0.25 g L^{-1}.

Furthermore, a drop in the pH was observed after a few days of growth (from pH 7.0 to 5.5-6.0), and was even more pronounced after one month of growth (less than 4) (data not shown). Usually, *T. claveryi* has been cultivated *in vitro* at pH 7, simulating its development under natural field conditions (alkaline soil) (Honrubia et al., 2014). To date, no substances have been added that buffered the pH during its cultivation but, according to our results, such a step is necessary since the fungal metabolism may produce organic acids from carbohydrates which are released into the medium promoting pH changes as reported by Rossi et al. (2007). The decrease in pH can also be explained considering that ammonium is transported into the fungus as ammonia, leaving the hydrogen ion behind (Griffin 1996). A slightly acidic pH is probably not unusual for a fungus that prefers neutral-basic soils, since *T. claveryi* hyphae have also to maintain a membrane potential by extruding H^+ ions around the plasma membrane in these soils.

Synergic effects of pH, carbon and nitrogen sources seen by response surface methodology

The experimental data obtained and those predicted by the model are compared in Table S1. The maximum mycelial biomass production was 4.65 g L^{-1}, which was observed with 15 g L^{-1} glucose, 0.5 g L^{-1} $(NH_4)_2HPO_4$ at pH 5, while the minimum level was 0.81 g L^{-1} with 10 g L^{-1} glucose, 0.75 g L^{-1} $(NH_4)_2HPO_4$ at pH 7. The residual ammonium was low in almost all treatments, indicating that this nutrient was consumed almost completely. The residual glucose was non-limiting, and part of it remained in the medium without being consumed (Table S1).

The analysis of variance (ANOVA) of the regression model was evaluated for biomass production (Table S3). The model was highly significant. Non-significant lack of fit (p-value > 0.05) is good for the model to fit. In this case, the p-value = 0.146 demonstrated that the quadratic model was highly significant. The R^2 value of the model was 0.42, indicating that 42% of the variance in the response could be explained by the model. The model also was significant for both residual glucose and NH_4^+ values and the non-significant values of lack of fit validated the models (Table S3).

The results of the regression analysis are shown in Table S4. The maximum biomass production was determined with optimal levels of pH (x_1), $(NH_4)_2HPO_4$ (x_2) and glucose (x_3) with multiple regression analysis to obtain a second-order polynomial equation expressed by Eq. 2, mainly considering the significant terms:

$$\boldsymbol{y_{biomass}} = 2.825 - 0.795x_1 + 0.541x_3 - 0.656x_2^2 - 0.756x_1x_3$$

where y_{biomass} is the biomass production, x_1 is the pH level, x_2 is the $(NH_4)_2HPO_4$ concentration and x_3 is the glucose concentration. The model reveals that only x_3 had positive effects on y_{biomass}, while pH, quadratic term x_2x_2 and interaction term x_1x_3 had negative effects (Table S4).

The 3-D response surface and contour plots of the combinated effects of glucose and $(NH_4)_2HPO_4$ levels on the biomass production at different pH levels were evaluated (Fig. 5). The model predicted 4.12 g L^{-1} of biomass production with 15 g L^{-1} glucose, 0.6 g L^{-1} $(NH_4)_2HPO_4$ and pH 5. Therefore, an increase in biomass production yield could be achieved by increasing the glucose concentration and lowering the pH. The $(NH_4)_2HPO_4$ behaviour was different, since it maintained its optimal value of 0.6 g L^{-1} (Fig. 5). The pH was efficiently buffered since it only decreased by approximately 0.2 units from the initial pH values. In fact, the buffer used was sufficient to maintain the pH values constant in the cultures. *In vitro,* several ECM fungi have shown a wide range of pH (between 3.5 and 8.5) to grow and to colonize plant roots properly (Sánchez et al. 2001). In *T. claveryi*, the strain T7 preferred a slightly acidic pH rather than a neutral one, under the conditions tested.

The carbon-nitrogen ratio (C/N) is an important factor affecting the mycelial development and fruiting body of medicinal mushrooms (López et al. 2011). Adjusting the optimal values for greater biomass, the C/N ratio was 25 (15/0.6) at pH 5, so that values above and below 30 were not optimal for mycelial growth. At pH 6, the mycelial growth was more stable with fewer variations regardless of the C/N ratio. Moreover, the best C/N for biomass production at pH 7 was 40, which it is similar to the ratio

279 found in the traditional MMN medium (10/0.25). The C/N relationships were conditioned by the pH of
280 the culture, where pH 5 favored biomass production compared with pH 7 (Fig. 5).

281 After 30 days, the bioreactor culture, in the optimized conditions, was harvested and a biomass
282 production of 3.1 g L^{-1} (Eq. 1) in dry weight was obtained. The linear consumption of 0.5 N NaOH
283 observed (Fig. S2) was probably necessary to compensate for the secretion of organic acids, products of
284 the metabolism associated with growth. The lag phase lasted 7 days, after which the mycelium began to
285 grow, the exponential phase lasting longer than in the flask culture. The biomass obtained was close to the
286 BBD predicted value, with a degree of accuracy of 75% of the model. Therefore, liquid fermentation in
287 bioreactors can be considered a suitable method for producing inoculum of *T. claveryi*, as long as the
288 need to optimize the conditions for each fungal strain has to be considered. Compared with other ECM
289 fungi, production of mycelium biomass of *T. claveryi* is much lower. In optimized culture conditions,
290 *Lactarius quieticolor* produced 3.25 g L^{-1} of biomass (0.11 g L^{-1} d^{-1}) and *Rhizopogon roseolus* produced
291 8.6 g L^{-1} (0.283 g $L^{-1}d^{-1}$) (Chávez et al. 2014). Rossi et al. (2011) obtained a productivity of 0.48 g $L^{-1}d^{-1}$
292 for the culture of *Pisolithus microcarpus*. In *T. melanosporum* fermentation, higher productivity was
293 achieved, reaching 1 g $L^{-1}d^{-1}$ (Liu et al. 2009). Whatever the case, *T. claveryi* production was 0.1 g $L^{-1}d^{-1}$
294 in the new growth conditions.

295 **Mycelial mycorrhization analysis**

296 The mycelium produced with the bioreactor was used for the production of desert truffle
297 mycorrhizal plants in the nursery, where the use of pure vegetative mycelial culture of ECM fungi is
298 probably the best method to inoculate plants (Morte and Honrubia 2009; Iotti et al. 2016). In addition, the
299 use of bioreactors for this purpose allows high-quality inoculum to be produced under controlled
300 conditions.

301 Although in natural field conditions *T. claveryi* with *H. almeriense* forms an endomycorrhiza, in
302 pot culture conditions it changes to an ectendomycorrhiza, and to an ectomycorrhiza with a typical sheath
303 and Hartig net under *in vitro* conditions (Gutiérrez et al. 2003), this type of mycorrhiza was redefined to
304 an ectendomycorrhiza *continuum* by Navarro-Ródenas et al. (2012).

305 Two-month-old micropropagated plants inoculated *in vitro* with *T. claveryi* mycelium were
306 analyzed and the results showed a good percentage of mycorrhization, (over 50% of the root system). A
307 high density of mycelium attached to roots, vermiculite and glass tube walls was observed under *in vitro*

conditions (Fig. 1e). Mainly, ectomycorrhizal colonization was observed, with roots surrounded by a typical sheath and intercellular hyphae (Hartig net) (Figs. 1f, g).

Mycelial growth of *T. claveryi* strains

Three of four additional strains tested did not grow, only strain T1 produced some extra biomass. It showed a significantly higher colony area and biomass in MMN-Optimized medium (4.51±0.15 cm^2 and 29.2±1.0 mg, respectively) than in MMN medium (2.97±0.20 cm^2 and 13.8±0.4 mg, respectively), according to Tukey's test ($p<0.05$). *T. claveryi* shows a very erratic growth over time as well as among subcultures, which makes it very difficult to draw general conclusions on the behavior of different strains.

In addition, the sexual reproductive mode of fungi should be taken into account for mycelial inoculations since, in heterothallic ascomycetes, the two MAT (mating type) genes occur in different strains, thus heterothallic ascomycetes are self-sterile, and the crossing between strains of opposite mating type is required for sporocarp production as demonstrated for *T. melanosporum* (Rubini et al. 2011). Up to now, it is unknown whether *T. claveryi* is heterothallic.

Conclusions

The macronutrients present in the MMN medium were not growth limiting and glucose was the best carbon source tested when combined with an appropiate initial amount of inoculum. A pool of added vitamins increased the mycelial biomass and the growth rate of *T. claveryi*. Carbon and nitrogen concentrations in the medium were adjusted to 15 and 0.6 g L^{-1}, respectively, and the pH set at 5 (MMN-Optimized) to improve the biomass production. Finally, 3.1 g L^{-1} of mycelial biomass was produced in the bioreactor by strain T7, thus providing a suitable amount of mycelium for large-scale mycorrhizal inoculation. These results constitute a valuable biotechnological advance for the continuous and efficient production of high quality desert truffle mycorrhizal plants. This work opens up the possibilities for providing enough amounts of mycelial inoculum of *Terfezia* strains for further studies on mycorrhizal efficiency and sporocarp production.

Acknowledgements

This work was supported by projects 19484/PI/14 (Fundación Séneca of Region of Murcia, FEDER, Spain) and CGL2016-78946-R (AEI-FEDER, UE). F. Arenas thanks the Ministerio de Economía y Competitividad (MINECO) for financial resources from the Youth Employment Initiative

(IEJ) and the European Social Fund (FSE) (PEJ-2014-A-83659). D. Chávez would like to thank the Chilean National Council for Science and Technology CONICYT 21110038 for the assigned postgraduate grant. A. Navarro-Ródenas is grateful to the University of Murcia for a postdoctoral contract.

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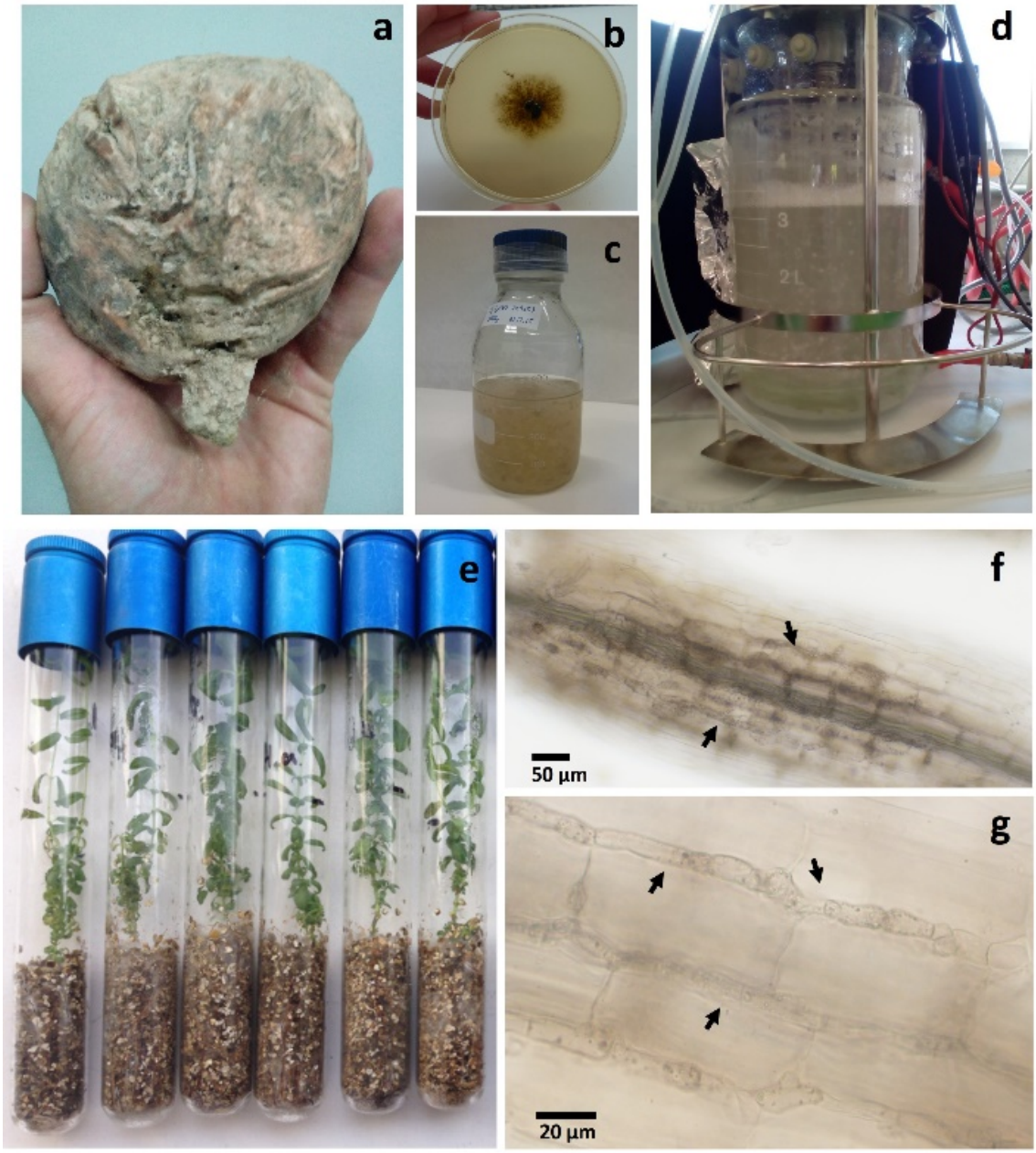


Fig. 1 Ascocarp of *Terfezia claveryi* (**a**), isolated mycelium of *T. claveryi* in MMN medium (**b**), mycelium preculture in liquid medium (**c**), fermentation process in a 5-L stirred tank bioreactor (**d**), mycorrhizal *H. almeriense* plants with *T. claveryi* liquid mycelium two months after *in vitro* inoculation (**e**), mycorrhizal colonization and Hartig net in stained roots under microscope are marked with black arrows (**f**, **g**).

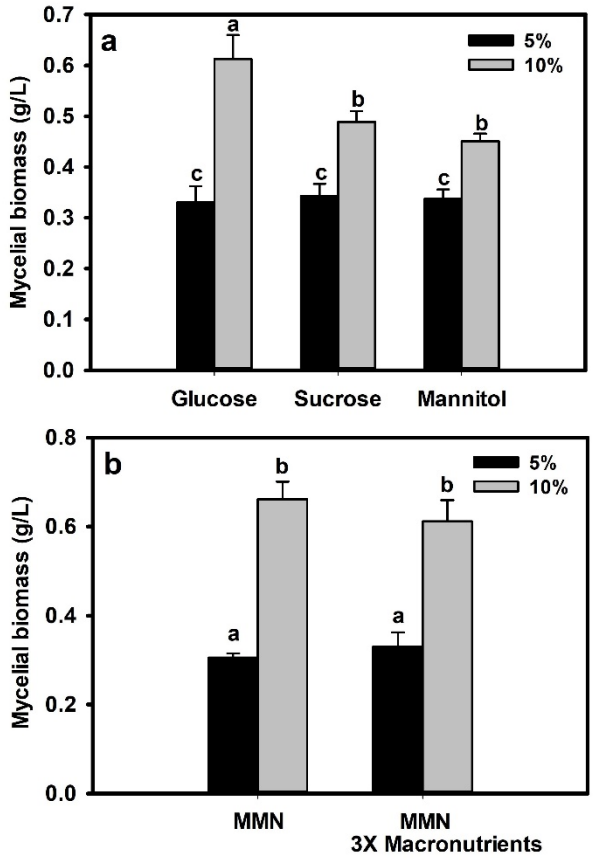
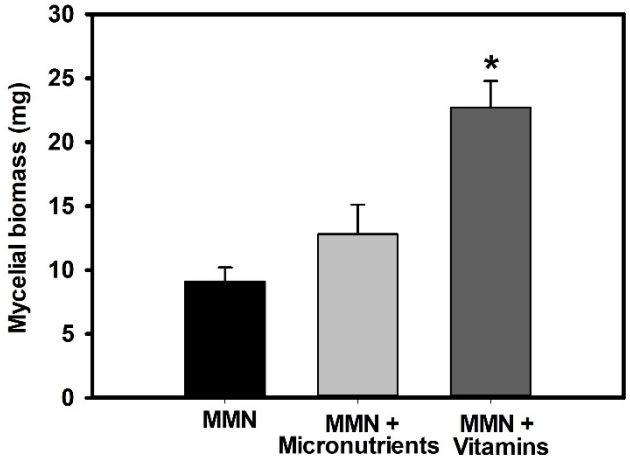


Fig. 2 Effect of different carbon sources (**a**) and effect of 3x [macronutrients] (**b**) on mycelial biomass of *T. claveryi* in liquid cultures with 5% and 10% of initial mycelial inoculum. Bars show the dry weight (g L^{-1}) means ± standard error (n = 5). Means followed by the same letter are not significantly different ($p<0.05$) according to Tukey's test.



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509 **Fig. 3** Effect of vitamins and micronutrients on mycelial biomass of *T. claveryi* on solid culture. Bars
510 show the dry weight (mg) means ± standard error (n = 15). The mean difference is significant at the
511 $P<0.01$ level according to Dunnett's test (*).

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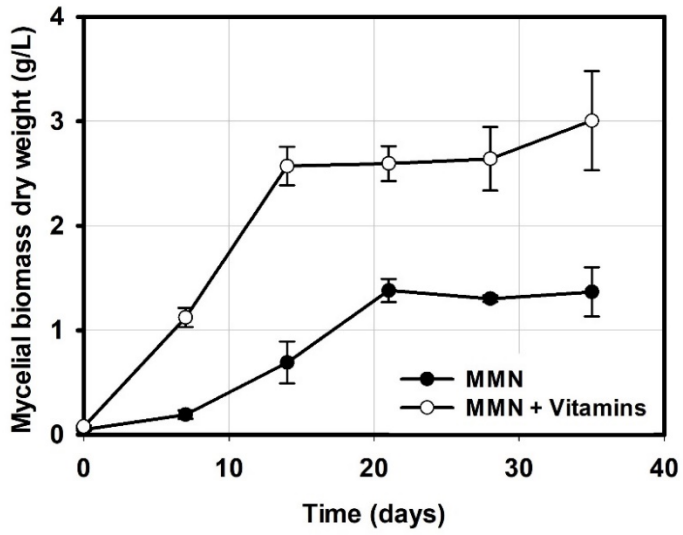

515

516 **Fig. 4** Mycelial growth (g L^{-1}) of *Terfezia claveryi* (strain T7) in MMN medium (black circles) and MMN
517 plus vitamins medium (white circles) in liquid culture. Values are the mean of (n=6). Bars indicate
518 standard error.

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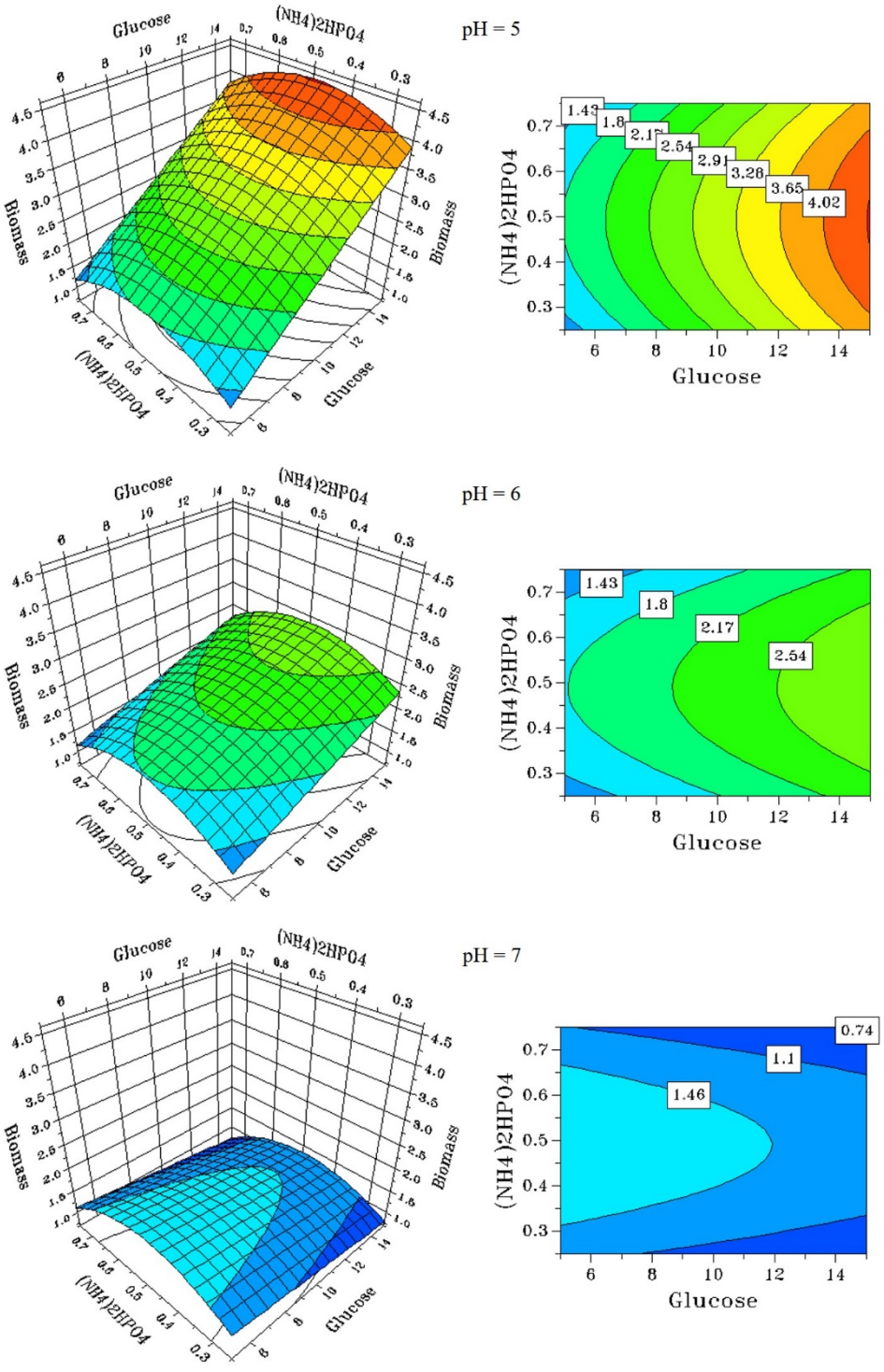

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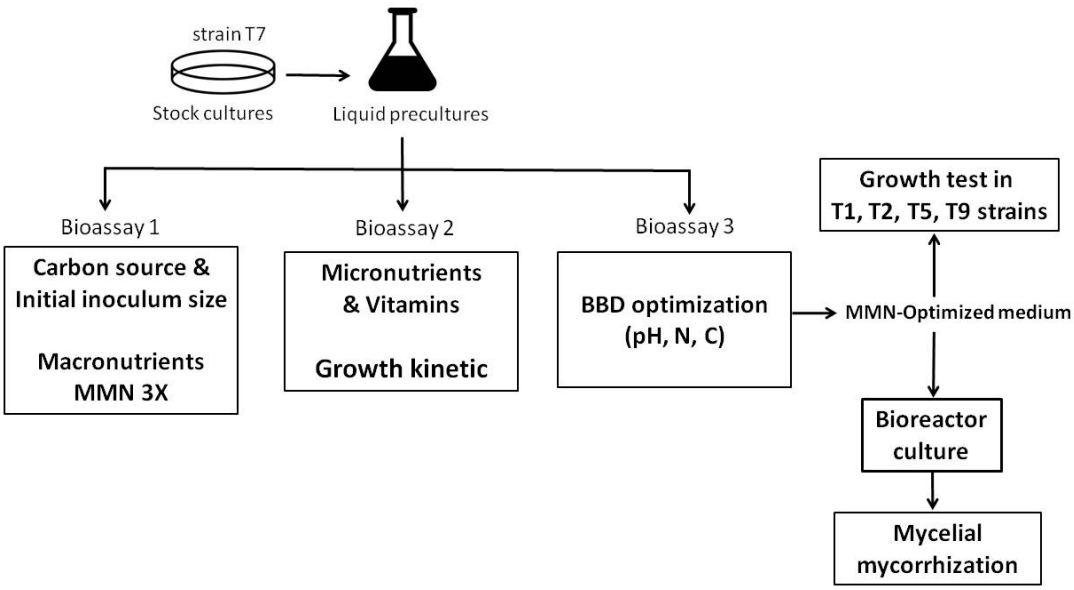
521 **Fig. 5** Response surface modeling for the synergic effects of different glucose and $(NH_4)_2HPO_4$
522 concentrations (g L^{-1}) on the mycelial biomass production (g L^{-1}) during liquid culture of *T.claveryi*. On
523 the right, 3-D contour plot and on the left, 3-D response surface plot. Top pH 5, middle pH 6 and bottom
524 pH 7.



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526 **Fig. S1** Summary of the different experiments performed.

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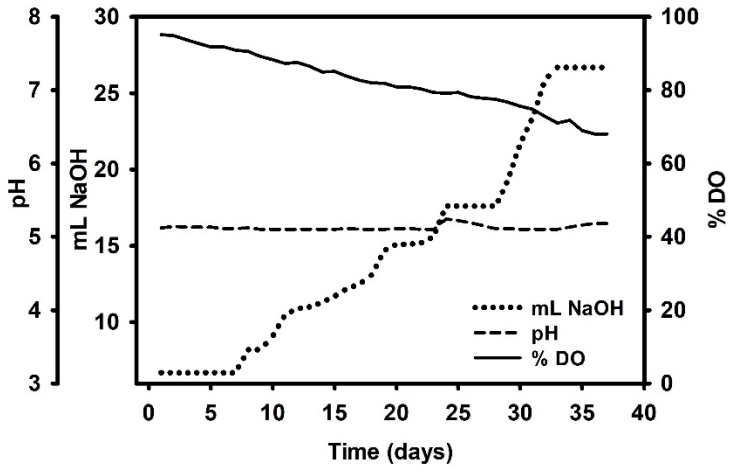

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532 **Fig. S2** Parameter profile during mycelial growth of *Terfezia claveryi* in bioreactor. Symbols for the

533 parameters used: pH (short dash), mL of NaOH added (dotted) and dissolved oxygen (%DO) (solid).