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IN VITRO HOST-FREE CALLUS DEVELOPMENT AND PLANT REGENERATION OF *OROBANCHE CRENATA* FORSK. AN OBLIGATE ROOT-PARASITE

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ABSTRACT

This study investigates the development of an in vitro system for callus induction, root-like protrusion formation, and plant regeneration in *Orobanche crenata*, a parasitic plant of economic significance to legume crops. The research aims to establish a controlled environment for studying *O. crenata* biology and physiology independent of host plants, offering insights into its growth dynamics and potential applications in parasitic weed management. Various culture media and growth regulators were tested to optimize conditions conducive to callus initiation and subsequent differentiation. Results demonstrate that B5 medium supplemented with gibberellic acid (GA) at 20 mg L-1 effectively induces callus formation and supports the development of root-like structures, crucial for further studies on host-parasite interactions and potential therapeutic applications.

Keywords: *Orobanche crenata*, in vitro culture, callus induction, root-like protrusions, plant regeneration, parasitic plants, growth regulators

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1. INTRODUCTION

The Orobanchaceae is a family of about thirty plant genera that develop haustoria and parasitize host plants through their roots (1). Extant species of Orobanchaceae represent an expansive range of parasitic lifestyles from photosynthetically competent genera like *Striga* to the non-photosynthetic *Orobanche* (2-3). in North Africa, the Near East and Western Asia (4). *Orobanche crenata* parasitizes Parasitic plants of the genus *Orobanche* comprise morae than 150 species (5); among them, seven (*O. crenata* , *O. cumana*, *O. ramosa*, *O. aegyptiaca*, *O. foetida*, *O. cernua*, and *O. minor*) are identified as noxious pests of economically important crops (4). *O. crenata* is considered the most widespread *Orobanche* of the Mediterranean region, particularly problematic mainly legume crops including faba bean (*Vicia faba*), chickpea (*Cicer arietinum*), pea (*Pisum sativum*), lentil (*Lens culinaris*), and various forage legumes (6-7). Infestation often results in a significant reduction of host yields (8-9) and as of yet there are no control methods that can be effective and economically employed against *O. crenata* on food legumes (10).

The life cycle of *O. crenata* is closely adapted to that of its hosts (11). There is both an underground development phase that starts with seed germination and growth of underground tubercles that subsequently develop roots and shoots, and an aerial step that begins from spike emergence and culminates with flowering and seed dispersal (5). A single *O. crenata* spike bear thousands of tiny seeds (0.2–0.3 mm in diameter), which are easily dispersed and remain viable in the soil for long periods (up to 15 years) (12).

On food legumes, none of control methods employed against O. *crenata* have proven to be both effective and economically feasible (10, 13). Furthermore, technical challenges related to the complexity of legume–parasite interactions represent a serious handicap to a better understanding of mechanisms involved in this host-*Orobanche* interaction. General features of host infection by parasitic plants are well known today, including aspects of *O. crenata* seed attachment and phenological stages during the development of the parasite (14) However, basic molecular knowledge of legume–*Orobanche* interaction is still limited due to several constraints, such as lack of axenic infection system that allow study of this interaction far from contaminations. *In vitro* culture techniques respond perfectly to these needs. Applied to parasitic weed, these techniques with optimized environment (temperature, light…) and nutritional conditions (growth medium, hormones…) allow i) easy, quick, and high frequency of regeneration; ii) development of pre-infection stages of the parasite in the absence of the host (15); and iii) homogenous parasite material like calli that could be used as source for host root infestation (15). Furthermore, these techniques could help for parasitic weed transcriptomic analysis, allowing the use of biological material exclusively from parasite without interference with host tissues (15-16).

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Interactions between *O. crenata* and legume hosts have included studies of parasite seed germination and parasitism of a host, but there are no reports of an *in vitro* system to investigate parasite development in the absence of a host (14, 17). An optimized *in vitro* growth system for *O crenata* will provide a method for regenerating plants in the absence of a host (15). Furthermore, *in vitro* culture may facilitate the collection of parasite oligo nucleotides, proteins or metabolites free from contaminating host materials (15).

Environmental conditions for the *in vitro* induction of callus from obligate parasitic weeds have been done on *Striga senegalensis* (18), *S. asiatica* (19), *cuscuta reflexa* (20), *O. ramosa* (5, 21), *O. crenata* (5), *O. aegyptiaca* (5), and *O. cumana* (5). Using a 2, 4 D and IAA regime Christopher et al (5) induced callus and the differentiation of root-like protrusions, but no further development was recorded. However, complete tissue culture system including plant regeneration from callus has been achieved only in few other *Orobanchaceae* species such as *Striga hermonthica* (22) and *Cuscuta reflexa* (20).

This suggests a potential limitation in the existing literature regarding the establishment of a complete tissue culture system, including plant regeneration from callus, in *Orobanche* species. The pursuit of *in vitro* culture techniques for *Orobanche* species serves multiple purposes. Firstly, it offers a controlled environment to study the biology and physiology of these parasitic weeds in detail, providing insights into their growth and development that may inform the development of targeted control measures. Within this framework, Ennami *et al* (23) has shed light on the genetic diversity of *Orobanche crenata*, revealing the existence of pathovars through the study of seven populations from different regions of Morocco using SRAP markers. This finding underscores the importance of *in vitro* culture techniques in elucidating the biological mechanisms underlying pathogenicity and host interaction in *Orobanche* species. By providing a platform for controlled experimentation, *in vitro* culture techniques allow researchers to explore the genetic basis of pathogenicity and resistance, paving the way for the development of targeted control strategies tailored to specific *Orobanche crenata* pathovars. Thus, the integration of *in vitro* culture techniques with genetic studies, such as those conducted by Ennami *et al* (23), represents a promising approach to advancing our understanding of *Orobanche* biology and improving management practices.

In this context, this study describes *in vitro* experimental system that allows control callus induction, development of root-like protrusions, and subsequent plant regeneration of *O. crenata* in absence of host plant. The system has been optimized using different growth media in association with different plant growth regulators.

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2. MATERIALS AND METHODS

2.1 Plant material

Orobanche crenata seeds were collected from mature *O. crenata* plants parasitizing faba bean fields at Marchouch experimental station of National Institute of Agriculture Research (INRA-Morocco). They were stored dry in the dark at 25°C.

2.2 Seed sterilization

Seeds were sterilized by sequential immersions with ethanol 70% (v/v) for two min, followed by 25% (v/v) of sodium hypochlorite solution containing Tween-20 (0.1%) for 15 min (all from Labox SA, Casablanca, Morocco). Finally, seeds were rinsed three times with sterile distilled water and air dried.

2.3 Viability test

Viability test was performed before and after sterilization using the modified 2,3,5-triphenyl tetrazolium chloride (TTC) method described by Van Hezewijk *et al* (24). Fifty mg of *O. crenata* seeds were placed in five ml of 1% TTC solution (Sigma) and incubated three days in the dark at 37°C. Seeds were examined using stereoscopic light microscope. Red or pink seeds were considered as viable, while, uncolored seeds were considered as dead.

2.4 Seed conditioning, germination and development

Dried aseptic *O. crenata* seeds were placed in Petri dishes containing autoclaved glass fiber paper (GF/A, Whatman) moistened with two ml of sterile distilled water. Seeds were maintained at 22°C to 23°C in the dark for at least 12 days before adding two mg L^{-1} of synthetic germination stimulant GR_{24} (obtained from Professor B. Zwanenburg, University of Nijmegen the Netherlands). Seedlings were grown on four different culture media containing gibberellic acid (GA) at 0 mg L^{-1}, 5 mg L^{-1}, 10 mg L^{-1}, 15 mg L^{-1}, and 20 mg L^{-1}. Overall, the selection of culture media and growth regulators was guided by the objective of optimizing conditions conducive to callus induction, root-like protrusion development, and plant regeneration in *Orobanche crenata*, thereby laying the groundwork for future experimentation and applications in parasitic weed management. The choice of culture media was informed by their composition and suitability for promoting cell proliferation and differentiation in *Orobanche*. Specifically, the selected media included: 1) Murashige and Skoog (MS) medium supplemented by MS vitamins (25); 2) Schenk and Hildebrandt (SH) medium supplemented by SH vitamins (26); 3) Gamborg's B5 medium supplemented by B5 vitamins (27), and 4) a modified Terrific Broth (TB) supplemented by TB vitamins (21) and containing 25 ml of macroelements (KNO_3, $Ca(NO_3)$·

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NH_4O_3, $MgSO_4$ $7H_2O$, Na_2SO_4, $NaH_2PO_4 \cdot 2H_2O$ and KCl); 2.5 ml of Microelements ($MnSO_4 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, H_3BO_3, $Na_2MoO_4 \cdot 2H_2O$, $C_uSO_4 \cdot 5H_2O$, and H_3BO_3); 25 ml of $CaCl_2$; and 2.5 ml of ferrique solution ($FeSO_4 \cdot 7H_2O$ and Na_2-EDTA $\cdot 2H_2O$). The first three media were purchased in commercially available powder from MB_{cell} (Los Angeles, U.S.A) while the last was made in the lab. All the four media were supplemented with sucrose and Myo-inositol. The pH was adjusted to 5.7 and the media solidified with 0.8% (w/v) of Agar–Agar. These media were chosen due to their well-established formulations and documented efficacy in supporting tissue culture and plant regeneration processes. Additionally, commercially available powder formulations from MBcell (Los Angeles, U.S.A) were selected to ensure consistency and reproducibility across experiments. Furthermore, the use of varying concentrations of Gibberellic acid (GA) as a growth regulator aimed to investigate its dose-dependent effects on callus induction and subsequent plant regeneration. Gibberellic acid is known for its role in promoting cell elongation and division, making it a suitable candidate for stimulating growth processes in *Orobanche crenata* tissues.

In the first experiment, *O. crenata* seeds germinated with GR_{24} were spread on three culture media (B5, MS, and SH) containing the five concentrations of GA (0 mg L^{-1}, 5 mg L^{-1}, 10 mg L^{-1}, 15 mg L^{-1}, and 20 mg L^{-1}). In the second assay, the GR_{24} treated *O. crenata* seeds were cultured on B_5 medium containing with growth regulator at different concentrations; namely GA (20 mg L^{-1}), Indole-3-acetic acid (IAA) (0.5, 1, and l.5 mg L^{-1}), and kinetin (Kin) (5, 10, and 15 mg L^{-1}).

For both assays plates were incubated in the dark at a controlled temperature (e.g., 20°C). Callus development was monitored using a stereoscopic light microscope to observe and record the morphology of developing calli at specified time points (e.g., 14, 21, 28, and 35 days after germination). For each media the number of calli induced over the total number of *O. crenata* seeds plated was determined. Callus morphology (color, structure, and size) were also recorded for each treatment. Optionally, for detailed morphological analysis, Calli were also photographed using a Cambridge Instruments Stereo-scan 120, Scanning Electron Microscope (SEM) (Cambridge, UK) at 15 kV. Image the callus samples using a suitable SEM instrument to visualize their microstructure at high resolution.

2.5 Statistical analysis

Statistical analyses in our study were conducted using the SPSS Statistics Software Program (version 17.0; SPSS, North Carolina, USA). Seven replications were performed for each assay to ensure robustness in our findings. We employed Analyses of variance (ANOVA) to assess the variations among treatments.

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Mean comparison was based on LSD (Least Significant Difference) multiple range classification tests, with significance defined at alpha =0.05. This post-hoc test allows for pairwise comparisons between treatment groups while controlling for family-wise error rate. Data handling was carried out using Microsoft office Excel (version 2007).

3. RESULTS

Our disinfection regime overcame the problem of eliminating contaminating microbes from the seed surface without reducing their germination rates (Fig. 1a and Fig. 1b). No significant difference of seed viability before and after sterilization was observed, with 91% and 89.4%, respectively.

Pre-conditioning treatment significantly enhanced the germination rate of *O. crenata* (Fig. 1c). Pre-conditioning for periods of 12 days followed by GR_{24} treatment resulted in germination rates of up to 63.45% whereas non pre-conditioning treatments resulted in significantly lower rates of germination 24.5%.

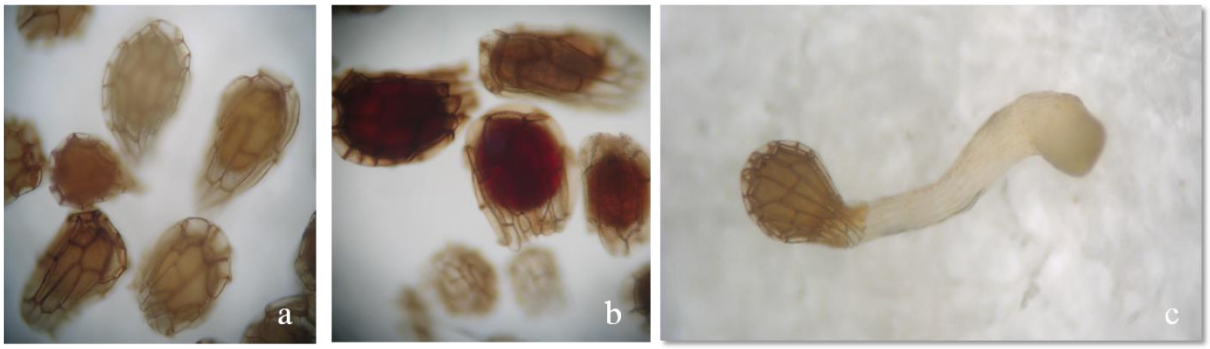

Figure 1: *O. crenata* seeds colored with TTC. (a): uncolored seeds were considered as dead. (b): red or pink seeds were considered as viable and (c) germinated *O. crenata* seed.

After seed conditioning, suitable incubation period of GR_{24} treatment, allowing optimal callus production depends on culture media used. Thus, this optimal duration was two days for B5 medium, with a maximum of 46.11% seeds developed into calli. Whereas, no developed calli was observed on MS and SH media. However, seven days of incubation allowed reaching 13.94% and 20.17% respectively (Table 1). Considering TB medium, no callus induction was observed during different time of incubation.

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Table 1: Effect of GR_{24} and culture media combined with GA on percentage of *O. crenata* callus induction. Results were obtained after 30 days of incubation with culture media

Medium	B5			MS			SH			TB		
GA (mg L^{-1})	2 d	5 d	7 d	2 d	5 d	7 d	2 d	5 d	7 d	2 d	5 d	7 d
0	43.66 ^{c}	4.6 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	15.86 ^{b}	0 ^{a}	0 ^{a}	0 ^{a}
5	36.68 ^{c}	16.2 ^{b}	0 ^{a}	0 ^{a}	3.47 ^{b}	4.08 ^{b}	0 ^{a}	0 ^{a}	20.16 ^{b}	0 ^{a}	0 ^{a}	0 ^{a}
10	34.52 ^{c}	17.31 ^{b}	0 ^{a}	0 ^{a}	2.81 ^{b}	5.18 ^{c}	0 ^{a}	0 ^{a}	16.18 ^{b}	0 ^{a}	0 ^{a}	0 ^{a}
15	30.78 ^{c}	19.49 ^{b}	0 ^{a}	0 ^{a}	3.04 ^{b}	11 ^{c}	0 ^{a}	0 ^{a}	9.17 ^{b}	0 ^{a}	0 ^{a}	0 ^{a}
20	46.11 ^{c}	18.25 ^{b}	0 ^{a}	0 ^{a}	7.23 ^{a}	13,94 ^{c}	0 ^{a}	3.43 ^{b}	20.17 ^{c}	0 ^{a}	0 ^{a}	0 ^{a}

Data represent means of 5 replicates. Means for the different days of incubation using GR_{24} (d) within a line followed by a different letter are significantly different at $P \leq 0.05$ according to LSD test.

To allow close observation of calli development, kinetics of *O. crenata* development was monitored during five weeks on three culture media (B5, MS, and SH). During the first stage of calli induction, undifferentiated cells were grown from developed radicle initiated by GR_{24}. At this stage, young calli were soft (Fig. 2a). This aspect evolved and changed over weeks depending on media and plant growth regulators.

Using MS and SH media with different GA concentrations, low callus induction rate was observed after 14 days of incubation, with a maximum of 2% and 4% respectively. These rates increase sharply over weeks. Thus, 35 days of incubation allowed reaching 17.54% of callus induction on MS medium with 20 mg L^{-1} of GA and 20.04% on SH medium with both five and 20 mg L^{-1} of GA (Fig. 3a, b). Considering Morphological appearance, *O. crenata* calli were slow growing, remained unorganized, carrying only amorphous protuberances on both MS (Fig. 2b) or SH media (Fig. 2c) with almost five different GA concentrations (Table 2). Except when MS medium contains 20 mg L^{-1} of GA, calli were soft, white-yellow, large, and a typical young shoot at the distal end of *O. crenata* calli were observed (Fig. 2d). Compared to MS and SH media, B5 medium combined with five different GA concentrations showed high calli induction rate, 15% of callus induction was recorded for two weeks of incubation and reached a maximum of 51.46% with 20 mg L^{-1} of GA after 35 days (Fig. 3c).

Furthermore, using B5 medium, calli were fast growing, showed much differentiation compared to MS and SH media (Table 2). Root-like protrusions were developed from calli only on B5 medium with both 0 mg L^{-1} (Fig. 2e) and 20 mg L^{-1} of GA (Fig. 2f), whereas, at remaining GA concentrations (5, 10, and 15 mg L^{-1}), calli were orange- brown, large, soft to hard and compact (Fig. 2f). Due to fast growth and differentiation of calli, B5 medium supplemented by 20 mg L^{-1} of GA was selected for subsequent experiment.

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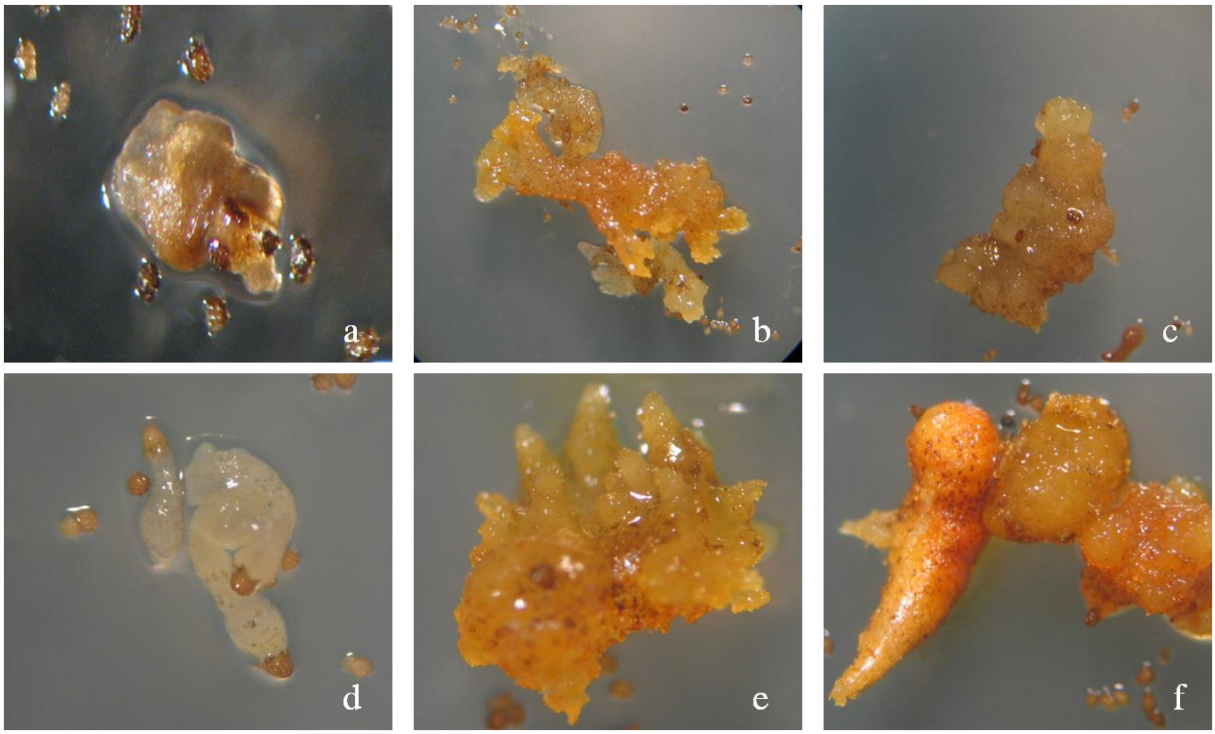

Figure 2: *In vitro* culture of *O. crenata* seeds on different culture media. (a): Initial stage of *O. crenata* callus development. (b): amorphous protuberances of *O. crenata*, grown on MS medium with 10 mg L^{-1} of GA. (c): amorphous protuberances of *O. crenata*, grown on SH medium with 15 mg L^{-1} of GA. (d): soft, white, large, and a typical young callus, grown on MS medium with 20 mg L^{-1} of GA. (e): root-like protrusions developed from *O. crenata* calli, grown on B5 medium without GA. (f): root-like protrusions developed from *O. crenata* calli, grown on B5 medium with 20 mg L^{-1} of GA.

Table 2: *O. crenata* Callus induction and morphology in relation with culture media (MS, SH, and B5) combined with 5 GA concentrations (0, 5, 10, 15, and 20 mg L^{-1})

Culture medium	Growth regulators (mg L^{-1})	Callus morphology
MS	GA-0	No callus induction
	GA-5	Small yellow callus.
	GA-10	Small yellow callus and some undifferentiated callus.
	GA-15	Small yellow callus and some undifferentiated callus.
	GA-20	Large callus, soft, white, and a typical young callus
SH	GA-0	Small yellow callus.
	GA-5	Small yellow callus.
	GA-10	Small yellow callus and some undifferentiated callus

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		GA-15	Small yellow callus and some undifferentiated callus
		GA-20	Small yellow callus and some undifferentiated callus.
B5		GA-0	Soft to hard, white to yellow, and some root-like protrusions.
		GA-5	Small callus, orange-brown, some differentiated callus
		GA-10	Large callus, soft to hard, orange-brown, some differentiated callus.
		GA-15	Large callus, soft to hard, yellow-orange, some differentiated callus
		GA-20	Large callus, soft to hard, orange-brown, and many root-like protrusions.
B5	GA-20	IAA-0.5 KIN- 5	Compact, yellow or brown, some root-like protuberances.
		IAA-0.5 KIN- 10	Compact, yellow or brown, some root-like protuberances
		IAA-0.5 KIN-15	Compact, yellow or brown, some root-like protuberances
		IAA-1 KIN-5	Compact, yellow or brown, many root-like protuberances.
		IAA-1 KIN-10	Compact, yellow or brown, many root-like protuberances.
		IAA-1 KIN-15	Hard-compact, faster-growing, yellow or brown, many root-like protrusions.
		IAA-1.5 KIN-5	Hard-compact, faster-growing, yellow or brown, many root-like protrusions
		IAA-1.5 KIN-10	Hard-compact, orange or brown, faster and differentiated callus
		IAA-1.5 KIN-15	differentiated calli, developed into shoot primordial, then into shoot-like structure comparable to *O. crenata* spikes

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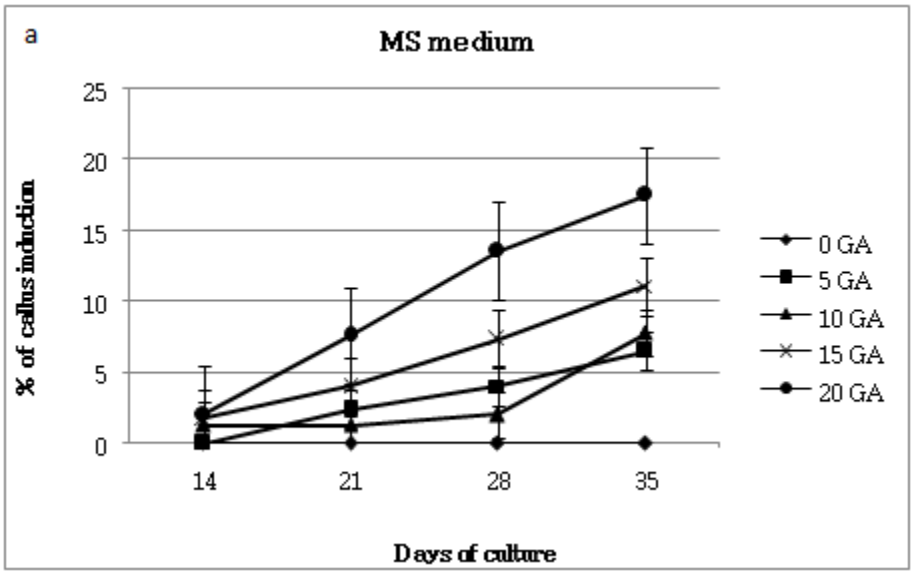

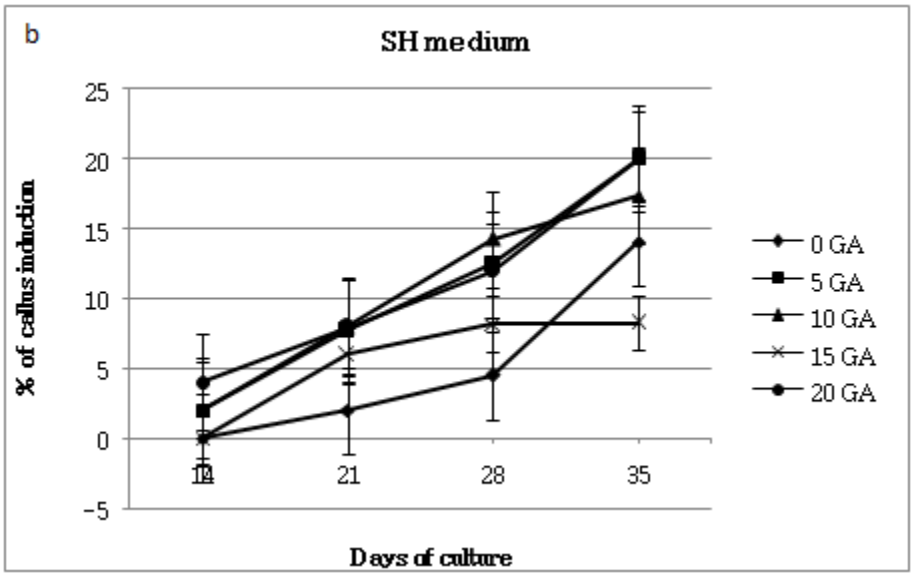

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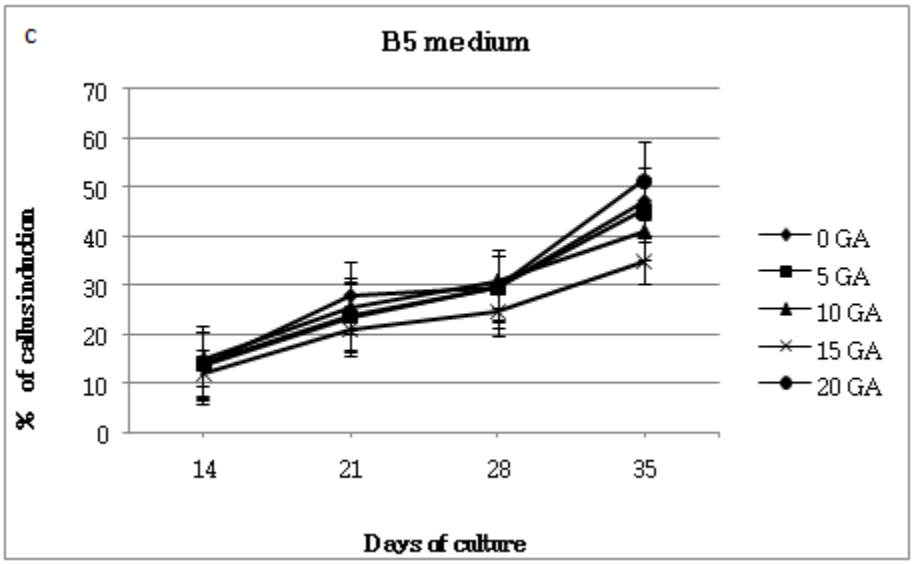

Figure 3: Percentage of *O. crenata* callus induction after being in culture on 5 concentration of GA (0, 5, 10, 15, and 20 mg L^{-1}) with three media. (a) MS culture medium, (b) SH culture medium, and (c) B5 culture medium. Data are means of 7 replications, with vertical bars indicating standard error.

The second assay was carried out on B5 medium supplemented with 20 mg L^{-1} of GA to investigate the combined effects of Indole-3-acetic acid and kinetin concentrations on callus differentiation of *O. crenata*, these combinations clearly influenced calli development. *O. crenata* calli induction occurred seven days after transferring seeds on growth media. Using combined concentration of IAA (0.5 and 1 mg L^{-1}) and KIN (5 and 10 mg L^{-1}) with 20 mg L^{-1} of GA, calli were hard, faster-growing differentiated organ as root-like protrusion. These structures were hard, either yellow or brown (Fig. 4a). SEM imaging confirmed structure of these thick root-like protrusions (Fig. 4b). Combining higher concentration of IAA (1.5 mg L^{-1}) and KIN (15 mg L^{-1}) with 20 mg L^{-1} of GA resulted differentiated calli (Fig. 4c) developed into shoot primordia (Fig. 4d), then into shoot-like structure comparable to *O. crenata* spikes (Fig. 4e and Fig. 4f).

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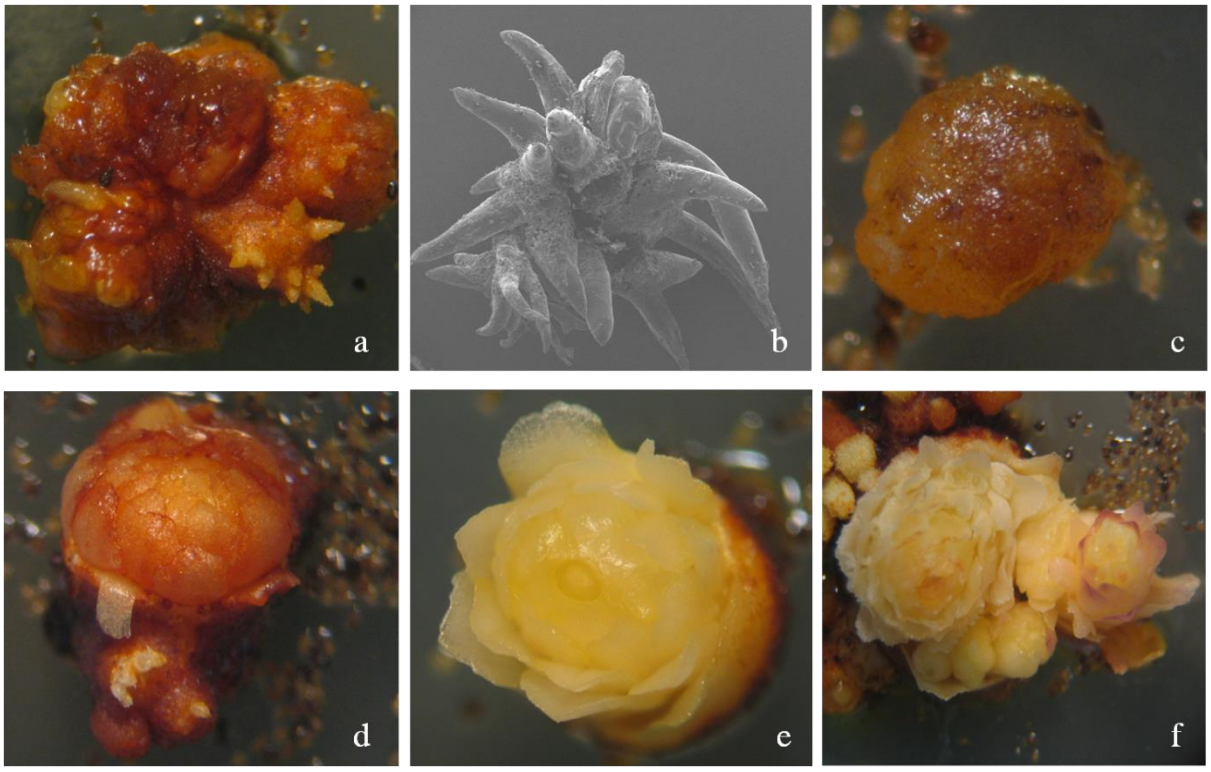

Figure 4: *In vitro* culture of *O. crenata* calli on B5 medium. (a): hard, faster–growing, and differentiated organ as root-like protrusion, grown on GA-20, IAA-1, and KIN-10. (b): Microscopy electron scanning of root-like protrusions. (c): differentiated calli grown on GA-20, IAA-1.5, and KIN-15 after 7 weeks. (d): shoot primordial grown on GA-20, IAA-1.5, and KIN-15 after 12 weeks. (e): shoot-like structure grown on GA-20, IAA-1.5, and KIN-15 after 17 weeks. (f): shoot-like structure comparable to *O. crenata* spikes grown on GA-20, IAA-1.5, and KIN-15 after 22 weeks.

4. DISCUSSION

The main goal of this work was to carry out a global study of *O. crenata* callus induction and tissue differentiation in *in vitro* system. Detailed callus differentiation studies for *O. crenata* have not yet been reported, except for one article (5). Thus, previous attempts to regenerate parasitic shoot, using sterile nutrient media and growth hormones, in absence of their host plant have been achieved only on *Striga hermonthica* (22), and Cuscuta reflexa (20).

Parameters adjusted in each step of this study, acts directly on optimization of calli induction and plant regeneration. Initially, *O. crenata* has been plagued by the seeds often being severely contaminated by soil micro-organisms (21, 25). For this reason, different methods of sterilization including the use of antibiotics or fungicides were tested, but none of them proved to eliminate

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contaminations and conserve seed viability (28-29). Therefore, our results show that our double surface disinfection technique using ethanol and sodium hypochlorite was appropriate to obtain axenic cultures of *O. crenata* while maintaining seed viability.

Pre-conditioning treatment (12 days) allowed high O. *crenata* seed germination rate (63.45%). Similar results were achieved by Ben-hod *et al.* (25), Zhou *et al.* (12), and Christopher et al. (5) where successful *Orobanche* seed germination was obtained after 10 and 14 days.

To allow *O. crenata* seed radicle emergence, GR_{24} was used as pre-treatment. On B5 medium, two days of incubation in presence of this synthetic germination stimulant was sufficient for high calli induction ranging from 30.78 to 46.11% regardless on the growth hormone concentration used. These results are in concordance with those of Zhou *et al*. (12); Christopher *et al*. (5) who reported that two days of incubation with GR_{24} was sufficient for calli induction of different *Orobanche* species using B5 media.

In our study, the percentage of callus induction and development depended on a combination of media and growth regulators. In fact, the first assay was carried out to report the effect of different culture media (MS, SH, and B5) with different GA concentrations on callus induction and development. We determined that B5 medium with 20 mg L^{-1} of GA initiated callus in 51.46%, of seeds, significantly higher than in MS or SH media. Similar results were observed by Christopher *et al* (5) who reported about 60 and 50% of *O. crenata* callus induction using B5 media combined with 2,4-D and IAA respectively. *O. crenata* callus induction was observed even in absence of GA when grown on B5 or SH media. Comparable studies reported the growth of *Striga asiatica* and *Striga gesnerioides* on simple, inorganic media with salts and sucrose but without growth regulators (30-31). However Christopher *et al* (5) reported contrasting results, in which B5 medium lacking plant growth regulators did not induce calli development of *O. crenata*, *O. aegyptiaca*, and *O. cumana*. Furthermore, using TB media, no callus induction was reported regardless of GA concentrations used, this is in contrast with results reported by Batchvarova *et al* (21) where combining TB media and GA succeed on inducing *O. ramosa* calli. Variation of the effectiveness of media on callus induction in different *Orobanche* species was described by Mangnus and Zwanenburg (32) and Westwood *et al* (33).

Gibberellic acid in combination with other plant hormones (IAA and KIN) has been extensively studied to enhance callus induction of different *Orobanche* species (12, 21). Our second assay allowed us to describe the calli types developed and degree of differentiation using combined three growth hormones within B5 medium. We observed root-like protrusions in B5 media containing IAA at 0.5 and 1 mg $L^{-1,}$ KIN at 5 and 10 mg $L^{-1}$and GA at 20 mg L^{-1}. Similar structures were observed on *Orobanche aegyptiaca* and *Orobanche ramosa* and these developed haustorial like connections with their hosts using IAA and GA (12). This author reported also

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that these root-like protrusions are a pre-request for host root infection by these *Orobanche* species through their conversion to haustorial connection. Use of sub-cultured calli from *O. crenata* unique seed will allow production of homogenous origin root-like protrusions that could be used for *in vitro* host infection. By doing so, intra-population variability of *Orobanche crenata*, reported recently by Ennami *et al* (14, 23) could be avoided and more accurate infestation results will be obtained.

Combining high concentration of IAA (1.5 mg L^{-1}), KIN (15 mg L^{-1}), and GA (20 mg L^{-1}) on B5 medium, allowed development of shoot-like structures. These structures have not hitherto by observed in *in vitro* culture of *O crenata*. Whereas shoots and root-like structures regeneration from callus has been achieved only on *Striga hermonthica* (22) and *Cuscuta reflexa* (20). While the chosen media and growth hormone combinations were effective in inducing callus formation and tissue differentiation in *O. crenata in vitro* system, the reliance on synthetic culture media and growth regulators may not fully replicate the natural environment of *O. crenata*. In fact, in nature, parasitic plants interact with their host plants and surrounding soil microbiota, which can influence their growth and development. By conducting experiments solely *in vitro*, the study may overlook important ecological factors that could impact the behavior of *O. crenata* in its natural habitat (34). Additionally, while the study focused on optimizing callus induction and tissue differentiation, it may not fully capture the complexity of *O. crenata* biology and ecology. Parasitic plants like *O. crenata* have evolved intricate mechanisms to locate and attach to host plants, extract nutrients, and complete their life cycle. These processes involve a multitude of genetic, physiological, and environmental factors that may not be fully represented in *in vitro* culture systems (2).

Recent parasitic weed transcriptomic analysis were carried out within three Orobanchaceae species (*T. versicolor*, *S hermonthica*, and *O. aegyptiaca*) on multiple stages of parasite development, starting from imbibed seed to parasite aboveground tissues (leaf, stem, and flowering spikes) in presence of host plants (35). It would be interesting to determine which *Orobanche* genes that are expressed at different stages *in vitro* during development from calli of differentiated organs such as r root-like and shoot like protrusions without interactions with host plants. Several studies have reported that *Orobanche* species can be rich in secondary metabolites, many with suspected pharmacological and antimicrobial activities (36-37). Optimizing *in vitro* culture system for *O. crenata* may provide a system for large-scale production of *O. crenata* tissue allowing possible economic exploitation of this plant's secondary metabolites.

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5. CONCLUSION

Our work aimed to determine optimal media and hormonal conditions for inducing callus growth and tissue differentiation in *O. crenata in vitro*. While previous attempts at regenerating parasitic shoots have been limited to other species, our study successfully established axenic cultures of *O. crenata* with maintained seed viability using a double surface disinfection technique. Our results indicate that the combination of GR_{24} and B5 medium is effective for callus induction, with varying rates depending on growth hormone concentrations. Particularly, we observed root-like protrusions and shoot-like structures under specific hormonal treatments, which present novel findings in *O. crenata* culture. Expanding upon the future directions of research in this area offers exciting possibilities for advancing our understanding of *O. crenata* biology and exploring its practical applications. Specifically, understanding gene expression dynamics during differentiation stages may shed light on the molecular basis of host-parasite interactions and the mechanisms by which *O. crenata* establishes connections with host plants. By identifying key genes and signaling pathways involved in these interactions, we can potentially develop targeted strategies to disrupt parasitic plant attachment and nutrient extraction, offering new approaches for controlling *O. crenata* infestations in agricultural settings.

Furthermore, exploring the potential economic exploitation of secondary metabolites from *O. crenata* tissue holds promise for various applications, including pharmaceuticals, agrochemicals, and biotechnology. Parasitic plants like *O. crenata* produce a diverse array of bioactive compounds as part of their adaptive strategies for parasitism. Investigating the biosynthesis pathways and bioactivity of these secondary metabolites could lead to the discovery of novel pharmaceuticals with therapeutic potential or environmentally friendly alternatives to synthetic agrochemicals.

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