CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

In vitro propagation of *Oxalis tuberosa*

BACHELOR'S THESIS

Prague 2020

Author: Martin Smetana

Supervisor: Ing. Iva Viehmannová, Ph. D.

Declaration

I acknowledge by my signature, that this bachelor's thesis is an original work written by me with the use of cited resources listed in references.

In Prague.....

Martin Smetana

Acknowledgements

I am sincerely grateful to my supervisor Ing. Iva Viehmannová, Ph.D., for her extraordinary patience, professional guidance and support in general whenever I needed help with this thesis or my work at the laboratory. She provided me with valuable advice and solutions even in times when it was not easy or during unexpected situations, such as the global coronavirus crisis during which the presented study was finished.

I would also like to thank the Faculty of Tropical AgriSciences and the Laboratory of plant tissue cultures for the possibility to work there and carry out all the experiments.

Last, but not least, my special thanks belong to my dearest friend Veronika Sekyrová for her support, to my boyfriend who survived all my complaints and to all my other friends and family members for their existence.

Abstract

Oxalis tuberosa Molina (Oxalidaceae) is a tuber bearing crop originating from the Andean mountain range in South America. The aim of this thesis was to develop a protocol leading to fast and successful *in vitro* micropropagation of this crop species. For shoot formation MS medium (Murashige and Skoog 1962) with plant growth regulators at different concentrations was used. 6–Benzylaminopurine (BAP) and zeatin (ZEA) were used either separately at concentration 0.1 mg l^{-1}, or in combination with α–Naphtaleneacetic acid (NAA) at concentration 0.03 mg l^{-1}. As control, MS medium without plant growth regulators was used. For *in vitro* rooting, MS medium containing NAA at concentrations of 0.1 or 0.3 mg l^{-1} was used. The most effective medium for shoot formation was the one containing 0.1 mg l^{-1} BAP, providing 11.30 shoots per plant, forming the highest number of meristems. The shoots rooted on all tested media, however optimal medium for rooting was the control reaching approximately 14.00 roots per explant in mean length of 2.08 cm. The micropropagation protocol developed in this study might be used to obtain high amounts of plants of *O. tuberosa* in a relatively short time period, and it can be used as a supportive technique for conservation and breeding purposes in oca.

Key words: Andean tuber crop; micropropagation; oca; plant growth regulator

Abstrakt

Oxalis tuberosa (*Oxalidaceae*) je hlíznatá okopanina pocházející z And v Jižní Americe. Cílem této práce bylo optimalizovat protokol mikropropagace této plodiny. Pro tvorbu výhonů bylo testováno MS médium (Murashige and Skoog 1962) s přídavkem regulátorů růstu v různých koncentracích. Cytokininy 6–benzylaminopurin (BAP) a zeatin (ZEA) byly použity buďto samostatně v koncentraci 0,1 mg l^{-1}, či v kombinaci s auxinem kyselinou naftyloctovou (NAA) v koncentraci 0,03 mg l^{-1}. K *in vitro* zakořeňování bylo použito MS médium obsahující 0,1 mg l^{-1} nebo 0,3 mg l^{-1} NAA. Jako kontrola bylo použito MS médium bez regulátorů růstu. Pro tvorbu výhonů u oky se nejlépe osvědčilo kultivační médium obsahující 0,1 mg BAP l^{-1}, na kterém rostliny vytvořily v průměru 11,30 výhonů na rostlinu, a zároveň nejvyšší počet meristémů. Získané výhony zakořeňovaly na všech testovaných médiích, avšak za optimální byla označena kontrolní varianta, s výsledkem 14,00 kořenů na rostlinu o průměrné délce 2,08 cm. Protokol mikropropagace optimalizovaný v této práci může být použit k efektivnímu klonovému množení rostlin *O. tuberosa* především pro účely uchování genetických zdrojů tohoto druhu a pro šlechtitelské účely.

Klíčová slova: andské okopaniny; mikropropagace; oka; regulátor růstu rostlin

Contents

List of tables

List of figures

List of abbreviations

BAP	6–Benzylaminopurine
NAA	α–Naphthaleneacetic acid
MS	Murashige and Skoog (1962) medium
PGR	Plant growth regulator
ZEA	Zeatin

1. Introduction

Oxalis tuberosa Molina, is a tuber bearing crop of the Oxalidaceae family originating from the Andean mountains in South America (Chase & Mark et al. 2016). It is a perennial herb of compact appearance, reaching up to 45 cm in height and 90 cm in width. It has succulent stems, clover-like leaves and small, yellow flowers. Tubers are edible, they are rich in starch, and they vary in color and shape depending on the genotype (King 1987).

This species is commonly known as oca in the Andean region (Valcárcel–Yamani et al. 2013) where it is grown and used mainly by the Andean communities. Like other Andean root and tuber crops, e.g. *Tropaeolum tuberosum* Ruiz & Pavón, *Ullucus tuberosus* Caldas, *Smallanthus sonchifolius* (Poepp. & Endl.) Robinson, etc., it is considered as underutilized crop (Brush 1995). Oca's relative hardiness in mountainous conditions (Leon 1964) makes it a great crop serving as a source of starches, vitamins and calcium (Ortega 1992).

It is difficult to obtain viable seeds due to the tri-stylous nature of oca flowers (Trognitz & Carrión et al. 2000). Polyploidy of this species could also be responsible for low seed production (White 1975). Farmers have propagated oca non–sexually by tubers for millennia to preserve the chosen traits in each variety (Pissard 2008).

Micropropagation as the most efficient method of vegetative propagation is a promising tool to obtain high amounts of plants in a short time. This technique could also be used as part of breeding and conservation programs in oca (George & Hall et al. 2008). So far, only primary studies focused on *in vitro* propagation of oca were carried out.

Thus, the aim of this study was to optimize a micropropagation protocol using nodal segments as original explants and different plant growth regulators (PGRs) at various concentration for *in vitro* shoot development and rooting of this species.

2. Literature review – *Oxalis tuberosa* Molina

2.1. Taxonomy and common names

There are about 800 currently known species in the *Oxalis* genus which is one of five genera in the Oxalidaceae family. The whole family is a part of the Oxalidales order which is a part of a huge clade of flowering plants known as Rosids (Chase & Mark et al. 2016).

Oxalis tuberosa Molina and its relative species form an unofficial group called *O. tuberosa* alliance. Some species in the alliance group are tuber bearing and *O. tuberosa* is one of them, also known as a tuber crop, called oca. Most species are not tuber bearing and are diploid, unlike oca (an octoploid species) and a few other species which show different ploidy levels. Even though several specimens of a wild form of oca were found in Bolivia (Emshwiller & Doyle 1998) it is unclear exactly which species are the ancestors of the domesticated oca or how its ploidy level changed, though it is thought that oca is an octoploid because of its hybrid origin (Emshwiller 2002).

Oca is a well-known crop among the local communities in the Andean regions of Peru and Bolivia, namely Quechua people, who have been cultivating oca for millennia there (Gibbs 1976). They call the tubers uqa which is where the most common name, oca, comes from. Other names include ibia, papa extranjera and cubio to name a few (King 1987).

2.2. Origin and geographical distribution

Oca is a species which is at home in the Andes, a South American mountain range, (Valcárcel–Yamani et al. 2013) and thus thrives in moist and cool mountainous conditions. It grows as high as 2,800–4,100 meters above the sea level. *O. tuberosa* does not tolerate frost very well and when exhibited to sub-zero temperatures, it dies. Periods of drought and higher temperatures are tolerated somewhat better but temperatures above 30 °C have the potential to harm the plants, just like the drought has. Ideal conditions for

oca to grow are humid with well distributed precipitation and temperatures between 5 and 17 °C. Suitable soil pH is 5.3 to 7.8 (Leon 1964).

Countries that lie in the area of oca distribution are mainly Bolivia, Peru and Ecuador which are the most important cultivation hot spots. Oca can also be found in other countries in the region such as Colombia but occasionally also in countries like Mexico (Pulgar 1981). Other species of the *O. tuberosa* alliance group are distributed mostly in mountainous areas from Costa Rica to Chile (Emshwiller 2002) (Figure 1). Outside of the Andes oca is a rare sight with New Zealand being the only exception as oca gained popularity there quickly and is cultivated under the local name "New Zealand Yam" (National Research Council 1989

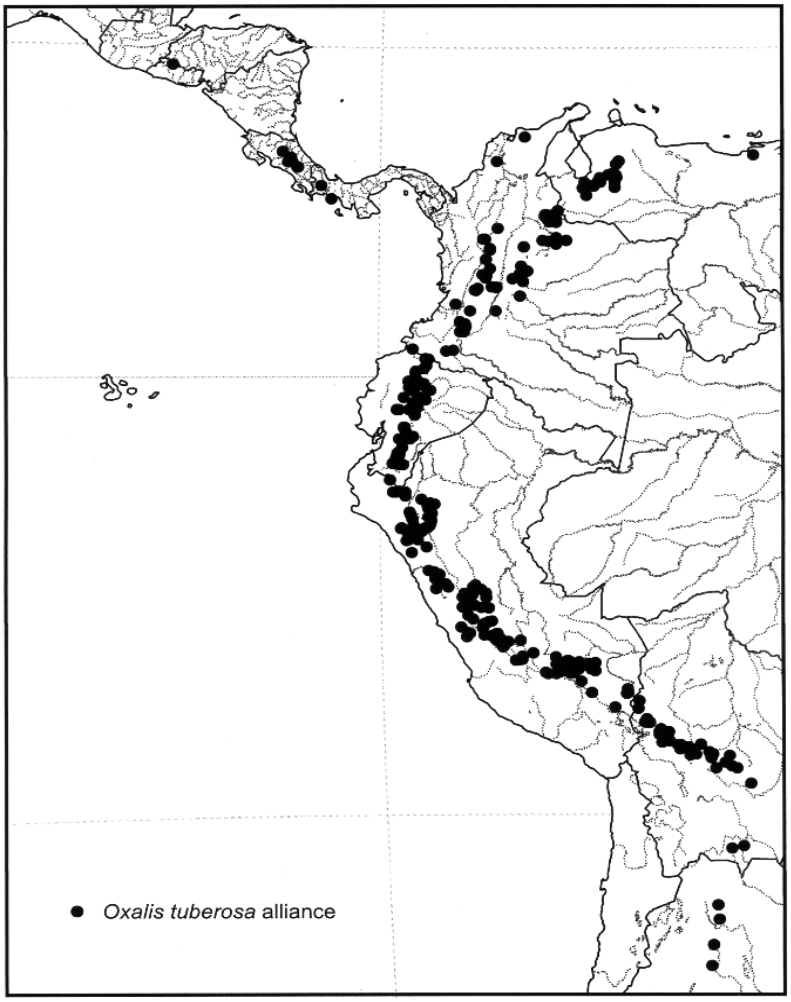

Figure 1 Distribution of *Oxalis tuberosa* alliance species in South America

2.3. Morphology

Oca plants have strong succulent stems which usually reach approx. 45 cm in height and spread up to 90 cm in width. Dark green leaves with plenty of trichomes resemble those of a clover (Figure 2) and grow in an alternate fashion (King 1987) According to Cardenas (1958) oca is prone to fasciation which can give the affected plant parts, usually stems and tubers, a very distinct appearance from normal. Oca, as many other *Oxalis* species, produces tri-stylous flowers which also results in hardened conditions for getting viable seeds (Trognitz & Carrión et al. 2000). This type of heterostyly means that oca produces 3 morphologically different types of flowers (one type in one specimen at a time) which are not freely interchangeable when it comes to fertilization which leads to a lessened probability of getting viable seeds if there are not the right morphotypes present at the same time. Another obstacle for successful seed collection is the fact that ripe and dried seed capsules are explosive and thus difficult to collect from the plant before seed dispersal happens (Cardenas 1958).

Figure 2 Oca leaves and flowers (Culbert 2012)

Roots are mostly thin and widespread. Tubers grow directly under the plant but can also be formed on stolons as far as 30 cm from the base of the plant. Though the biggest tubers can reach 20 cm in length and 4 cm in width, more often they are approximately a half of that size. Color of the tubers depends on the grown variety of oca and is very variable (Figure 3), e.g. pink, red, violet, white, yellow or almost black. Shape

of the tubers is as variable as the color, with uneven surface and smooth or rough (King 1987).

Figure 3 Oca tubers (Linksvayer 2012)

2.4. Chemical properties, use and importance to humans

Oca tubers are very rich in easily digestible carbohydrates (sucrose and starch) and as such are a good source of energy (Gross et al. 1989) while the content of fiber is rather low (Janick & Simon 1988). It also has a higher content of certain essential amino acids, namely valine and lysin (1.0 and 0.065 mg g^{-1} of fresh weight, respectively). The tubers were found to contain substantially higher amounts of iron and phosphorus (0.8 and 34.0 mg g^{-1} of fresh weight) than potatoes. Thus, oca tubers are an important source of nutrients as oca is at least as nutritious as potato, or even more (National Research Council 1989). According to Cortes (1977), oca could be used commercially to produce flour, alcohol and starch. Other than that, oca is a great source of vitamins and calcium (Ortega 1992).

Oca has many uses in the traditional cuisine and overall an essential role in the local agricultural systems. For example, the tubers can be used as boiled, baked, dried, freeze–dried, fermented etc. An old freeze-drying technique is used to make a typical local dish among the Quechua people which is called chuño. It is usually prepared during the coldest months of the year (July and August) when the temperature drops below zero

during the night but raises again during the day thus drying and freezing the tubers successfully. Then the tubers acquire a unique flavor can be stored for about 10 years (Bradbury & Emshwiller 2011).

Oca and other Andean tuber crops are endangered by underutilization. Also, the local communities often do not practice the old customs and traditions anymore which also means that the knowledge of cultivation of the traditional crops is being lost (Brush 1995).

2.5. Ecological requirements, cultivation and propagation

2.5.1. Ecological requirements

Oca is very humble when it comes to soil requirements. It grows well in poor soils; the only fertilizer which is suitable is compost or other complete organic fertilizer. Industrial fertilizers with nitrogen promote growth of the top plant part but not really the size of the tubers. Soils with pH in range of 5.3–7 (mildly acidic–neutral) are the best (Leon 1964).

Oca has a potential to be introduced as a new crop in areas with suitable climate conditions namely in Asia and Africa (for example Nepal and Rwanda) but also North America (mainly the Pacific Northwest due to late first frost dates) (King & Gershoff 1987). Oca shares the cultivation areas with potatoes (*Solanum tuberosum*) but unlike potatoes, oca is hardier in terms of resisting to lower temperatures and soil nutrients (Pulgar 1981). The potential of oca as a new crop for many temperate zone areas would be achieved by selecting traits like lower oxalate content in tubers or better frost hardiness (Leon 1964).

As some species of the *O. tuberosa* alliance grow in rather dry environment of western slopes of the Andes while others in humid cloud forests in hilly areas it is theoretically possible to use these species with different properties and breed them with oca to make the crop more resilient and productive in various environments. Although this subject requires further research (Emshwiller & Doyle 1998).

2.5.2. Cultivation

A month before oca is planted the chosen field should be deeply plowed to bury post-harvest remnants from previous season and to eliminate weeds and pests. It is essential to ensure a good drainage of the plot because oca tubers could rot from excess of water. Planting methods vary a lot among farmers and some still traditionally plant oca in combination with other tuber crops such as ullucus or mashua, but monocultures seem to prevail as the yields tend to be higher. Planting oca together with beans or corn is also practiced. The "seed" tubers do not necessarily have to be collected but the ones left in the soil from the last harvest can be used and many farmers do so because oca does not store well , especially when the tubers are damaged or placed in moist environment. But some farmers do choose which tubers they will plant next after everything is harvested. The best quality tubers are either traded or consumed, the second-grade ones are used for planting and the worst are fed to pigs. An optimal "seed" tuber weights 30–40 grams and it is recommended to plant 750 kg of tubers per hectare. Compost and fermented manure are recommended as fertilizers, crop rotation (with potatoes, corn and legumes) is also recommended to keep soil fertile (Suquilanda 2011). The tubers usually start forming approximately 105 days after sprouting, gaining and are ready to be harvested in another approximately 100 days (Alarcón 1968). Depending on the environmental conditions they can gain up to 6.6 g of mass a day (Rivero 1976). With 25,000 plants ha^{-1} a harvest of 35–57.5 t ha^{-1} (Figure 4) can be expected depending on the density of planting, spaces between rows should be 50–80 cm wide and plants 37–50 cm from each other (Jiménez 1986).

To some communities in the Andes oca is even more important than potato. For example community of Sacaca in Cusco region of Peru grows oca, potatoes and other tuber crops at the elevation of 3,400 meters above the sea level and their oca yield is 18 t ha^{-1} a year compared to only 5.4 t ha^{-1} of potatoes or 13.2 t ha^{-1} of ullucus. The difference in yields of traditional cultivation systems compared to more conventional farming methods is noticeable (Morlon & Rivera Martínez 2014).

Oca can be negatively affected by pests or diseases. In some areas, beetle larvae (*Systena sp.*) cause damage, in other the damage come from fungal pathogens such as *Fusarium sp.* or *Rhizoctonia sp.* This can be prevented by choosing resistant varieties for the risky regions (Gonzáles et al. 2003).

Figure 4 Farmers with harvested oca in La Paz, Bolivia (LEISA magazine 2014)

2.5.3. Propagation

In the Andean traditional agricultural systems, oca is propagated in a vegetative way by tubers. By this it is ensured that farmers will get the same oca variety every year. The bigger the tuber the faster it is possible to get a full-grown plant. When grown from seeds, oca plants can appear as of the same variety but still can be of different genotypes, thus using tubers for propagation is encouraged. In the period of growth before the formation of tubers, oca can also be propagated from stem and stolon cuttings. The cuttings can be rooted in water or moist soil (Pissard 2008).

Sexual reproduction in oca could theoretically be used by farmers to obtain a new variety which would then be propagated from tubers (Trognitz & Hermann et al. 1998) although this method is not traditionally used by the farmers (Gibbs 1976, Trognitz et al. 1998). It is also not easy to acquire viable oca seeds due to the tri-stylous flowers (Trognitz & Carrión et al. 2000). As the tubers used to propagate oca could also be used for consumption, an application of biotechnological methods for effective vegetative propagation, e.g. micropropagation, would be beneficial.

2.5.4. Micropropagation

Micropropagation in general is a technique of vegetative propagation utilized for scientific, commercial and hobby purposes. The plants undergoing the process of micropropagation are grown in sterile containers such as glass flasks in a controlled environment of a laboratory. The plant tissue cultures can be started from seeds or it can be obtained by surface sterilization of explants which are plant body parts containing meristems and thus allowing to propagate, regenerate and get new viable plants through simple regeneration or somatic embryogenesis. Plants grown *in vitro* are nourished from the growth medium which contains and supplies the plant with every nutrient, moisture and serves as an anchoring substrate essential to a healthy growth. Plant growth regulators can be added as well. This method of propagation is relatively rapid and is mainly to obtain large quantities of genetically identical material (George & Hall et al. 2008).

For several species of the *Oxalis* genus, process of micropropagation has been already optimized, e.g., for *O. variifolia* Steud. and *O. gracilis* Jacq., South African species grown as ornamental plants. In an experiment implemented by Crouch and van Staden (1994) MS medium (Murashige and Skoog 1962) was used supplemented with different PGRs in different treatments. The first treatment contained α–Naphtaleneacetic acid (NAA) and kinetin. The second treatment contained NAA and 6–benzylaminopuryne (BAP). The result showed that this propagation method, especially the kinetin treatment, can be used as mean of propagation of these ornamental *Oxalis* species.

Specifically, in oca, use of micropropagation is also possible yet scarce with successful results. As the starting material apical or axillar buds can be used, as well as buds (“eyes”) found on tuber surface (Mejía–Muñoz et al. 2006). According to Khan (1988) use of PGRs such as a synthetic cytokinin BAP or auxin NAA is recommended in oca micropropagation. Among other factors a photoperiod of 16 hours and 26 °C seem to promote balanced growth suitable for micropropagation. According to the research on oca by Mejía–Muñoz et al. (2006) the tuber buds prove to be a useful source for micropropagation with use of PGRs (BAP and NAA). Micropropagation in oca can be a useful method for conservation or breeding purposes.

In the present study, the goal is to optimize the micropropagation process in oca with the use of nodal segments as the initial explants and the comparison of two

cytokinins BAP and ZEA either with or without the addition of auxin NAA at low concentration. Further, rooting with the use of NAA at 2 concentrations is tested. A successful outcome of this study would provide a guideline for mass propagation of oca in a relatively economically favorable way.

3. Aims and hypotheses of the thesis

3.1. Aims

The main aim of the thesis was to develop a micropropagation protocol for Andean tuber crop, *Oxalis tuberosa*, i.e., to optimize *in vitro* propagation and rooting of this crop.

Based on the main aim the following partial objectives were defined

- to establish an *in vitro* culture of *O. tuberosa*
- to optimize *in vitro* propagation phase using nodal stem segments
- to root *O. tuberosa* shoots

3.2. Hypothesis

The objectives of the thesis were based upon the following hypothesis

- NaClO will be efficient for surface sterilization of plant material
- induction of new shoots will be improved by addition of cytokinins BAP and ZEA in culture medium
- rooting of shoots will be improved using auxin NAA in culture medium

4. Materials and methods

4.1. Plant material

For the experiment, *O. tuberosa* originating from Peru was used. This plant material was maintained in the botanical garden of the Faculty of Tropical AgriSciences (FTA). The tubers were pink on the surface and the pulp was yellow pink.

4.2. Methods

4.2.1. Establishment of *in vitro* culture and initial multiplication of plant material for the experiment

The experiment was carried out in the Laboratory of Plant Tissue Cultures of the Department of Crop Sciences and Agroforestry at the Faculty of Tropical AgriSciences of the Czech University of Life Sciences Prague in 2018/2020.

The stems from the original plants growing in the FTA botanical garden were cut into apical and nodal segments. Then the cuttings were sterilized using 70% ethanol for 10 seconds and then 2% NaClO for 5 minutes. After this procedure the cuttings were rinsed 3x in sterile distilled water. The dead tissue was cut off before the segments were introduced to a sterile basal MS medium containing 100 mg l^{-1} myoinositol, 30 g l^{-1} sucrose and 10 g l^{-1} agar. The pH was adjusted to 5.7 and then the medium was sterilized with the use of autoclave for 20 min at 121 °C.

The plants were grown under 36 μmol m^{-2} s^{-1} fluorescent light with day/night cycle of 16 hours of light and 8 hours of darkness. The temperature was 23 °C at dark and 26 °C during the light period. Newly grown shoots were separated as plants were subcultured every 2 weeks on the same medium.

After the plant culture was successfully established it was necessary to obtain sufficient plant material for the *in vitro* experiment. As the plants did not produce offshoots to be conveniently propagated on basal MS medium, they were transferred on

an MS medium with addition of plant growth regulators BAP and NAA, both at concentration of 0.1 mg l^{-1}. The plant shoots, before being used for the experiment, then were transferred to a basal MS medium where they grew taller and in uniform manner.

4.2.2. Micropropagation of *O. tuberosa*

The experiment was established using uniform, three-nodal stem segments without leaves which were cut off (Figure 5). For *in vitro* propagation, following treatments were used: MS + 0.1 mg l^{-1} BAP, MS + 0.1 mg l^{-1} BAP + 0.03 mg l^{-1} NAA, MS + 0.1 mg l^{-1} zeatin, MS + 0.1 mg l^{-1} zeatin + 0.03 mg l^{-1} NAA. As a control, MS medium without PGRs was used. There were 20 explants per treatment. The initial explants for the micropropagation experiment measured 0.3–0.5 cm in length.

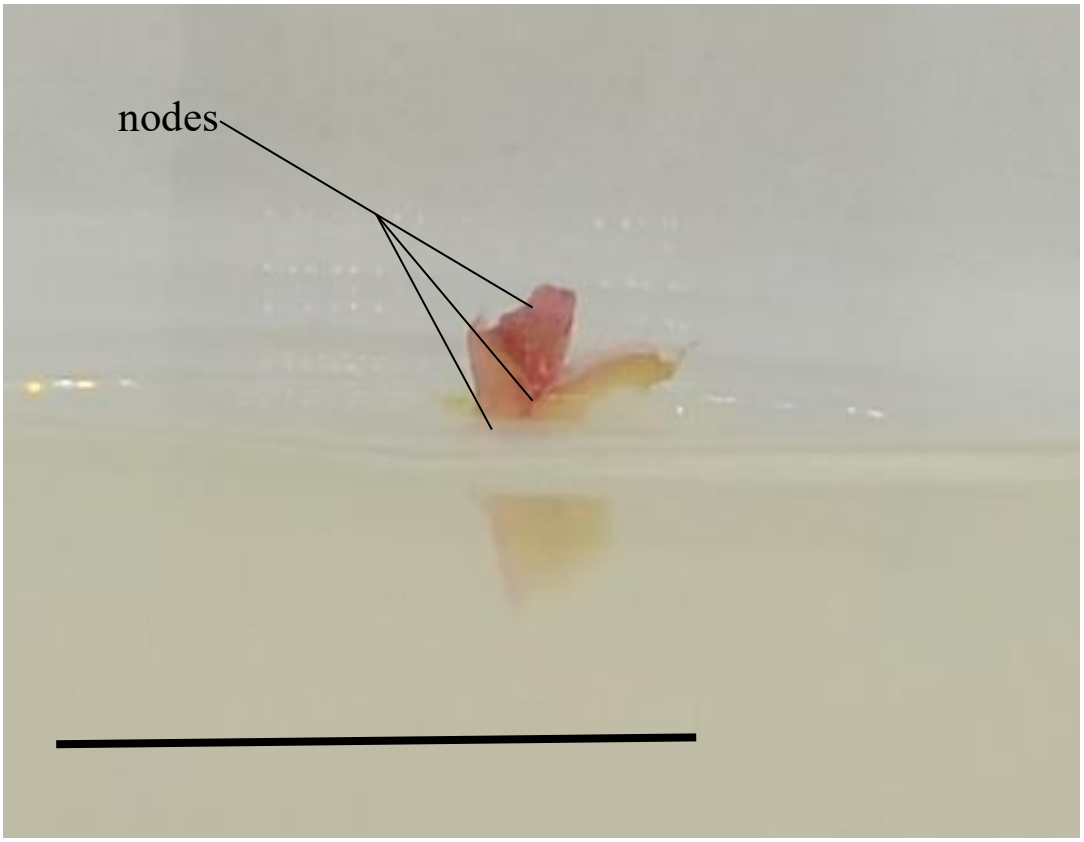

Figure 5 The initial explant (bar = 1.25 cm) (author)

On these media, plants were cultivated 3 weeks. After this time period, the plants were evaluated. The measured characteristics included number and height of shoots (≥

0.3 cm), number of leaves, meristems (axillar + apical shoot counts) and roots (≥ 0.3 cm). Conditions of the cultivation were as described above.

4.2.3. *In vitro* rooting of *O. tuberosa*

In case of rooting, basal MS medium with the addition of NAA at concentrations of 0.1 mg l^{-1} or 0.3 mg l^{-1} was used. There were 20 explants per treatment. Shoots at mean length of 0.5 cm from the BAP treatments with the mean count of 5 meristems per shoot were chosen to establish the *in vitro* rooting experiment. The experiment was evaluated after 3 weeks. The number of roots per explant and the length of the roots was measured.

4.2.4. Statistical evaluation of collected data

After collection of all the data from the experiments, evaluation by the statistical analysis was carried out (STATISTICA 12.0 software by StatSoft, Inc.). One-way ANOVA (analysis of variance) was used followed by Tukey's HSD test ($p \leq 0.05$) to determine statistically significant difference between the resulting means.

5. Results

5.1. Establishment of *in vitro* culture

Approximately 20% of the initial explants used for *in vitro* sterilization remained undamaged, not contaminated and alive. The rest did not survive either due to the sterilization process (15%) or the following fungal contamination (85%).

The surviving explants started to regrow within a week of cultivation. The original shoots kept growing longer but did not form any additional shoots, thus stem segments were used for further propagation. On the medium supplemented with 0.1 mg l^{-1} BAP and NAA the stems segments started producing new shoots from nodal meristems. Overall, most new shoots were produced from meristems on the basal part of the segments. These shoots were used for further propagation on the same medium, till sufficient plant material for the experiment was obtained. Due to irregular growth of shoots on this medium, they were transferred on MS medium (for 3 weeks) before being used for the experiment.

5.2. *In vitro* propagation

The explants on the both BAP supplemented media showed overall better results than those on the control medium and plants on media supplemented with ZEA (Table 1).

First visible growth of shoots, regardless of medium used, was noticed within a week of cultivation. The new shoots grew from all 3 nodal meristems of the original explants and these shoots kept producing other shoots from their meristems which resulted in vigorous branching (11.30 shoots per explant in 0.1 mg l^{-1} BAP only and 12.00 shoots per explant in 0.1 mg l^{-1} BAP + 0.03 mg l^{-1} NAA). This plentiful multiplication was not observed in the ZEA treatments or in control. Thus, the treatment supplemented with 0.1 mg l^{-1} BAP was regarded as the best one as it used the cytokinin alone.

On media supplemented with BAP, the shoots were relatively short but also produced more meristems in comparison to other treatments. Thus, the plants appeared

to be very compact, with minimal spaces between nodes (Figure 6 b, c). In terms of shoot formation and their length there was no statistically significant difference between the plants cultivated on the medium supplemented with 0.1 mg 1^{-1} BAP only and the one with addition of 0.03 mg l^{-1} NAA. The NAA supplemented medium resulted in a slightly higher number of shoots per explant and their slight prolongation, however, the number of meristems was lower. Thus, the BAP only treatment was regarded as the better one as the addition of NAA did not bring any significant benefits. No roots or callus were observed in none of the media with addition of BAP.

The two media supplemented ZEA showed slightly better shoot formation than the control (2.85 shoots per explant in 0.1 mg l^{-1} ZEA only and 2.75 shoots per explant in 0.1 mg l^{-1} ZEA + 0.03 mg l^{-1} NAA) but the resulting numbers of shoots and meristems were not significantly different from the ones in the control. The shoots grew from the 2 top nodes or all 3 but there was no further branching. The shoots in both ZEA treatments were significantly longer than those in the BAP treatments but much shorter and thicker than the ones in the control (Figure 6 d, e). As in the BAP treatments, addition of 0.03 mg l^{-1} NAA had no statistically significant effect on the growth and development of explants. Unlike in the BAP treatments, in ZEA treatments it was observed that plants on the NAA-free medium, produced higher numbers of shoots and meristems than on the medium with NAA. Like in the BAP treatment with NAA the shoots were longer on the medium with ZEA and the addition of NAA. No roots or callus were observed in none of the media with addition of ZEA.

The control group of plants produced the lowest number of shoots (1.95 per explant) and meristems though these results were not significantly different from the results of the ZEA treatments. The shoots were mostly produced by the first 2 nodes on top of the explant rather than the one at the base. No branching occurred. The shoots produced were by far the longest and thinnest (Figure 6 a) among all the treatments. This was also the only group of plants which produced roots (Figure 6 f). No callus was observed in the control medium.

Table 1 Results of *in vitro* propagation of *Oxalis tuberosa*

Basal medium	PGRs (mg l^{-1})	Shoots per explant (≥ 0.3 cm) (mean ± S. E)*	Length of shoots (cm) (mean ± S. E)*	Leaves per explant (mean ± S. E)*	Meristems per explant (mean ± S. E)*	Roots per explant (≥ 0.3 cm) (mean ± S. E)*
MS	0	1.95 ±0.11 b	3.90 ±0.30 a	17.40 ±0.90 b	19.40 ±0.90 b	6.90 ±0.41
MS	0.1 BAP	11.30 ±0.67 a	0.51 ±0.02 c	51.05 ±2.44 a	62.35 ±2.81 a	n/a
MS	0.1 BAP + 0.03 NAA	12.00 ±0.64 a	0.56 ±0.03 c	48.00 ±3.36 a	60.00 ±3.69 a	n/a
MS	0.1 ZEA	2.85 ±0.08 b	0.87 ±0.04 b	19.90 ±0.93 b	23.25 ±1.00 b	n/a
MS	0.1 ZEA + 0.03 NAA	2.75 ±0.10 b	1.05 ±0.05 b	21.45 ±0.97 b	24.20 ±1.00 b	n/a

*S. E = standard error
Data of the same column followed by the same letter were not significantly different according to Tukey's HSD test ($p \geq 0.05$)

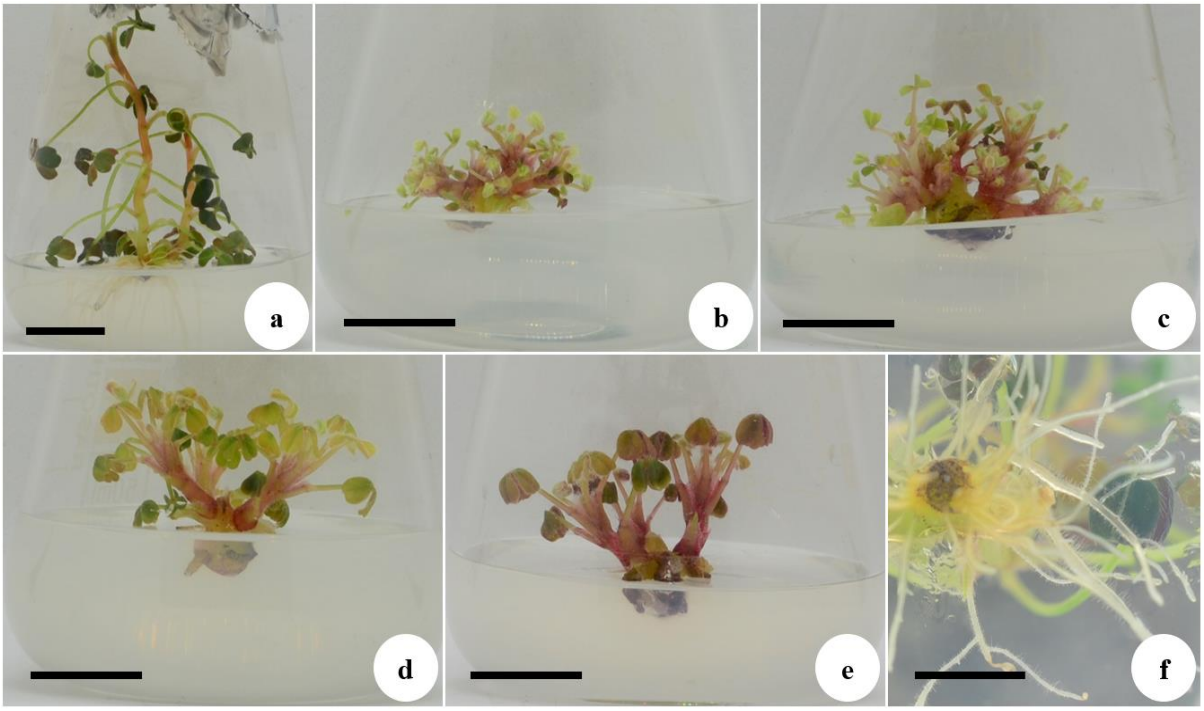

Figure 6 Oca explants after 3 weeks of cultivation on MS medium. **a** PGR-free control (bar = 1.25 cm). **b** 0.1 mg l^{-1} BAP (bar = 1.25 cm). **c** 0.1 mg l^{-1} BAP + 0.03 mg l^{-1} NAA (bar = 1.25 cm). **d** 0.1 mg l^{-1} ZEA (bar = 1.25 cm). **e** 0.1 mg l^{-1} ZEA + 0.03 mg l^{-1} NAA (bar = 1.25 cm). **f** Roots developed on plants cultivated on the PGR-free control (bar = 0.6 cm). (author)

5.3. *In vitro* rooting

None of the *in vitro* propagation treatments produced roots, and thus, auxin NAA at two concentrations (0.1 mg l^{-1} and 0.3 mg l^{-1}) added in MS medium was tested.

The first roots were visibly forming at the end of the first week of cultivation, and after 2 weeks they became stronger and single roots were clearly recognizable. The most rapid growth followed during the third week of the experiment. All the roots apparently started growing at the same time.

There was no significant difference in number of roots per explant among the tested NAA concentrations and control (Table 2). However, there was a significant difference in the length of the roots in the NAA treatments and control. Both NAA treatments produced shorter roots than the control. The roots were shorter with higher NAA concentration although not significantly (Figure 7 b, c). The roots in the control treatment were significantly longer and extensively branching (Figure 7 a). Plants of the control treatment in this experiment produced higher number of roots per shoot than the three nodal segments cultivated on control MS medium in previous experiment focused on *in vitro* propagation.

Table 2 Results of *in vitro* rooting of *Oxalis tuberosa*

Basal medium	PGRs (mg l^{-1})	Number of roots per explant (≥ 0.3 cm) (mean ± S. E.)*	Length of roots (cm) (mean ± S. E.)*
MS	0	14.00 ± 1.28 a	2.08 ± 0.08 a
MS	0.1 NAA	14.70 ± 1.11 a	0.76 ± 0.04 b
MS	0.3 NAA	14.15 ± 1.45 a	0.70 ± 0.03 b

*S. E = standard error
Data of the same column followed by the same letter were not significantly different according to Tukey's HSD test ($p \geq 0.05$)

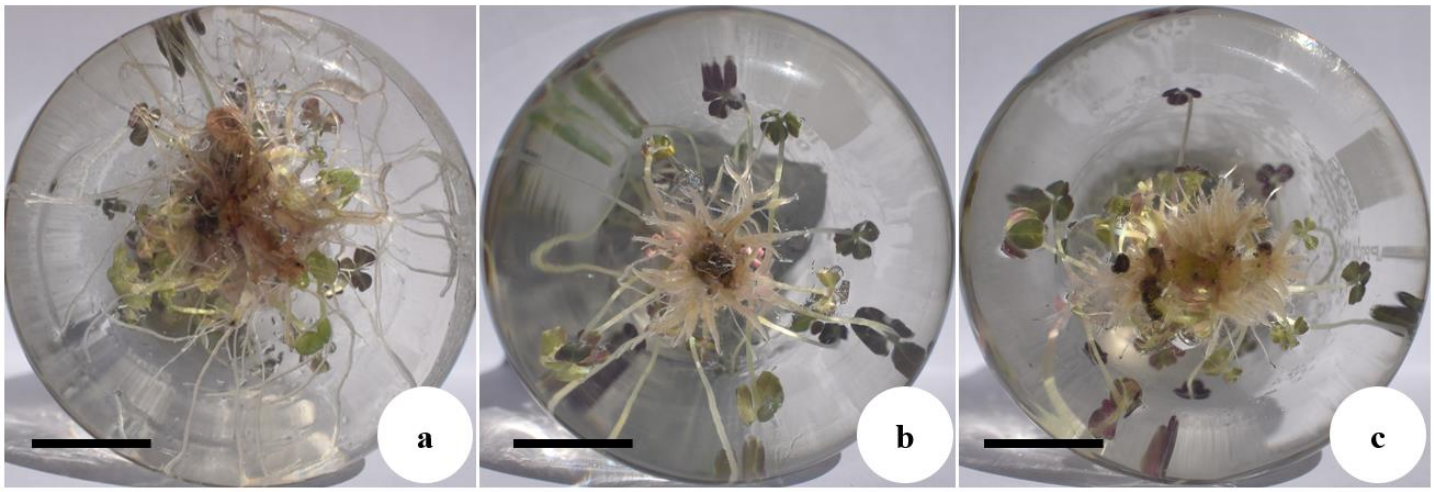

Figure 7 Oca explants after 3 weeks of rooting on MS medium supplemented with NAA. a NAA-free control (bar = 1.1 cm). b 0.1 mg l^{-1} NAA (bar = 1.1 cm). c 0.3 mg l^{-1} NAA (bar = 1.1 cm). (author)

6. Discussion

6.1. *In vitro* propagation

In the present study, explants of *O. tuberosa* cultivated on MS medium supplemented with 0.1 mg l^{-1} BAP showed the best results in meristem formation leading to approximately 62 meristems per explant, however, the treatment with 0.1 mg l^{-1} BAP and 0.03 mg l^{-1} NAA did not provide significantly different results. Cytokinin BAP alone and in combination with auxin NAA proved to be successful for *in vitro* shoot regeneration of some *Oxalis* species (*O. reclinata*, *O. helicoides*) (Crouch & van Staden 1994) and apparently do positively influence shoot regeneration in *O. tuberosa* as well, our results show. Mejía–Muñoz et al. (2006) in their study on *in vitro* multiplication of oca reported that MS medium supplemented with 1 mg l^{-1} BAP also produced 12 shoots per explant, which suggests that the increased concentration of BAP does not essentially lead to better results, as comparable results were obtained in our study using 0.1 mg l-1 BAP. Apparently, neither effect of NAA used in our experiments had significant effect on shoot proliferation despite the fact that in some cases a synergic effect of NAA and BAP was observed in other plant species (Alawaadh et al. 2020).

BAP commonly shows improvement in shoot formation up to a certain concentration and at higher concentrations causes decrease in it, although in some species use of lower concentrations seems to deliver a better response in shoot formation such as in the micropropagation of *Aloe polyphylla* Pillans (Bairu et al. 2007). The effect of BAP promoting better shoot formation up to a certain concentration has been observed in other studies with herbaceous plants for example in micropropagation of *Stevia rebaudiana* Bertoni where 1 mg BAP l^{-1} was the most effective concentration while higher ones led to decrease in shoot formation (Rokosa et al. 2020) or in micropropagation of 2 banana cultivars (*Musa acuminata* Colla) where 2 mg l^{-1} BAP was the most successful concentration and the shoot formation rate only decreased at higher concentrations (Selvakumar & Parasurama 2020).

Another reason responsible for such differences in growth could be the use of a different plant material as there were significant differences in oca genotypes in the study

carried out by Khan et al. (1988) or the fact that explants from different parts of the plant tend to behave differently (Yaacob 2012). Mejía–Muñoz et al. (2006), for example, used buds of oca tubers obtained from farmers, while in our experiment, three nodal segments were used.

The shoots formed in both BAP treatments in our study were relatively short and were branching extensively which is similar to effects of BAP observed in other plant species, e.g. *Solanum lycopersicum* Linnaeus (Danial & Ibrahim 2018).

No callus development was observed in the BAP or ZEA treatments, probably due to the relatively low concentration of the cytokinins and NAA as callus is often produced by explants when higher hormone concentrations are used (Devi et al. 2008). This effect was observed in the genus *Oxalis* according to the study of *O. glaucifolia* Knuth and *O. rhombeo–ovata* A. St. Hil. (Escandon et al. 1989) and in oca as well (Khan et al. 1988). According to some studies, the use of BAP can lead to hyperhydricity in explants (Grigoriadou 2020) or their abnormal growth (Kubalakova & Strnad 1992). Nevertheless, neither of these issues was noticed in our study.

Zeatin is considered to be a very potent cytokinin and therefore is, despite its high price (Peixe 2007), widely used in micropropagation often in combination with auxin NAA to promote shoot formation (Borgato & Furini). Our results, however, suggested that this might not be the case in *O. tuberosa*, as the two ZEA treatments resulted in only slightly better shoot and meristem formation rate in comparison to control. Even though the shoots were slightly longer in the ZEA treatment with NAA the difference in comparison to the ZEA only treatment was not significant. Contrary to our study on oca, zeatin exhibited better results than BAP in shoot formation of several exemplary species: tomato (*Solanum lycopersicum* Linnaeus) (Pawar 2012), strawberry (*Fragaria* x *ananassa* Duch.) (Debnath 2006) and vanilla (*Vanilla planifolia* Jacks. ex Andrews) (Giridhar & Ravishankar 2004). Despite the fact that zeatin might seem to be highly effective in most cases, BAP provides better results in some plant species, (e.g. *Chlorophytum borivilianum* Sant. & Fern.) (Khatri et al. 2019).

According to the results of the presented study it can be assumed that oca is significantly more sensitive to the stimulating effect of BAP on cell division and lateral bud growth (Gaspar et al. 1996) than that of ZEA.

When the number of nodes in the control treatment in our study is compared to other treatment the data support the fact that even though shoots in the control treatment were the longest, they also developed the smallest number nodes but with relatively long internodes which corresponds with the results obtained on a medium without any PGRs in a study on *Solanum lycopersicum* (Danial & Ibrahim 2018). In the present study, oca formed the highest number of nodes with relatively small distances between them in both BAP treatments. In both ZEA treatments the number of nodes was smaller but the distances between nodes were larger. This phenomenon shows a different sensitivity of oca to different cytokines at the same concentration and their stimulation properties of cell division and lateral bud growth induction (Gaspar et al. 1996). The great number of shoots and meristems results in plant material which is optimal for micropropagation (Conner et al. 1993). The results imply that use of BAP in micropropagation of *Oxalis tuberosa* leads to satisfactory results in shoot regeneration.

6.2. *In vitro* rooting

The addition of NAA in rooting media at concentrations of 0.1 or 0.3 mg l^{-1} did not promote significantly better rooting in comparison to the control. This finding was similar to the result of the study on *Solanum lycopersicum* carried out by Danial & Ibrahim (2018) in which it was assumed that the naturally high content of auxins in the plant body is responsible for the ineffectiveness of NAA.

Although the difference in length and growth of the roots between both NAA treatments and the control in the present study was significantly different, the number of roots developed was not. The longest and branched roots were obtained from the control while the NAA treatments yielded shorter ones and were sparsely branching which is on the contrary with usual effects of NAA that is believed to promote root elongation (Ghanti et al. 2004). However, in some plants NAA promotes thickening of the roots rather than elongation (Vuylsteker et al. 1998), this observation is consistent with the results in our study on oca. Plants rooted on basal MS also developed longer shoots which is considered beneficial allowing for easier manipulation, especially when *ex vitro* transfer follows. Hence, use of basal MS without any PGRs is suggested as the most economically plausible and efficient way.

7. Conclusion

The applied surface sterilization (70% ethanol for 10 seconds and then 2% NaClO for 5 minutes) proved to be effective in introduction of the plant material into *in vitro* culture.

For *in vitro* shoot production in oca, MS medium supplemented with 0.1 mg l^{-1} BAP proved to be the most efficient. This treatment allows to propagate oca in large quantities as the average number of regenerated shoots was 11.30 and number of meristems was 5.52 per shoot. Even though the BAP treatment supplemented with 0.03 mg l^{-1} NAA formed slightly more shoots, the meristem count was lower and the treatment with BAP only was evaluated as the most successful and economically convenient one. However, ZEA did not prove to be efficient sufficiently in induction of new shoots.

For rooting of the shoots, optimal medium was the control one, basal MS without any PGRs. The presence of NAA at concentrations 0.1 or 0.3 mg l^{-1} did not cause formation of more roots than the control. The roots in NAA treatments were shorter and not branching unlike in the control. Considering that the addition of NAA did not improve rooting in oca, therefore basal MS medium can be recommended as optimal and cheap option. Thus, according to our results NAA did not proved to be efficient in improving rooting in *O. tuberosa*.

This micropropagation protocol could be used in order to produce larger quantities of *Oxalis tuberosa* plants for scientific, conservation or breeding purposes.

8. Recommendation

Micropropagation protocol developed in this study was optimized using one clone of oca, and it can serve as a first step for application and possible modification of this procedure for other genotypes. Within future research activities, wider range of PGRs and their combinations might be tested for both, *in vitro* propagation and rooting. Optimization of *ex vitro* transfer is also a crucial step of a micropropagation procedure.

9. References

Alarcon M. 1968. Ritmo de tuberización en cinco clones seleccionados de oca. Tesis. UNSAAC, Cusco, Perú.

Alawaadh AA, Dewir YH, Alwihibi MS, Aldubai AA, El–Hendawy S, Naidoo Y. 2020. Micropropagation of lacy tree *Philodendron* (*Philodendron bipinnatifidum* Schott ex Endl.). Horticultural Science 55: 294–299.

Bairu MW, Stirk WA, Dolezal K, Van Staden J. 2007. Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: can meta–topolin and its derivatives serve as replacement for benzyladenine and zeatin? Plant Cell, Tissue and Organ Culture 90: 15–23.

Borgato L, Pisani F, Furini A. 2007. Plant regeneration from leaf protoplasts of *Solanum virginianum* L. (Solanaceae). Plant Cell, Tissue and Organ Culture 88: 247–252.

Bradbury EJ, Emshwiller E. 2011. The role of organic acids in the domestication of *Oxalis tuberosa*: a new model for studying domestication resulting in opposing crop phenotypes. Economic Botany 65: 76–84.

Brush SB. 1995. In situ conservation of landraces in centers of crop diversity. Crop Science 35: 346–354.

Cardenas M. 1958. Estudios sobre tubérculos alimenticios de los Andes. Comunicaciones de Turrialba, Costa Rica.

Chase MW et al. 2016. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Botanical Journal of the Linnean Society 181: 1–20.

Conner AJ, Zhang X, Wooding AR. 1993. Micropropagation of oca on a high sucrose medium promotes starch accumulation and plant establishment in soil. New Zealand Journal of Crop and Horticultural Science 21: 91–93.

Cortes H. 1977. Avances en la investigación de la oca. Congreso Internacional de Cultivos Andinos, Ayacucho: Serie de Cursos y Conferencias y Reuniones No. 178. Instituto Interamericano de Cooperacion para la Agricultura. Lima. 45–48.

Crouch NR, van Staden J. 1994. *In vitro* propagation of a number of South African *Oxalis* species. South African Journal of Botany 60: 134–135.

Danial GH, Ibrahim DA. 2018. New protocol of Tomato (*Lycopersicon esculentum* Mill.) *in vitro* propagation. Revista Innovaciencia 6: 1–13.

Debnath CS. 2006. Zeatin overcomes thidiazuron-induced inhibition of shoot elongation and promotes rooting in strawberry culture *in vitro*. The Journal of Horticultural Science and Biotechnology 81: 349–354.

Devi R, Dhaliwal MS, Kaur A, Gosal SS. 2008. Effect of growth regulators on *in vitro* morphogenic response of tomato. Indian Journal of Biotechnology 7:526–530.

Emshwiller E, Doyle JJ. 1998. Origins of domestication and polyploidy in oca (*Oxalis tuberosa*: Oxalidaceae): nrDNA ITS data. American Journal of Botany 85: 975–985.

Emshwiller E. 2002. Ploidy levels among species in the *Oxalis tuberosa* Alliance as inferred by flow cytometry. Annals of Botany 89: 741–753.

Escandon AS, Hopp HE, Hahne G. 1989. Differential amplification of five selected genes in callus cultures of two shrubby *Oxalis* species. Plant Science 63: 177–185.

Gaspar T, Kevers C, Penel C, Greppin H, Reid DM, Thorpe TA. 1996. Plant hormones and plant growth regulators in plant tissue culture. In Vitro Cellular & Developmental Biology – Plant 32: 272–289.

George EF, Hall MA, De Klerk G. 2008. Micropropagation: Uses and methods. Plant propagation by tissue culture. Springer, Dordrecht, The Netherlands.

Ghanti K, Kaviraj CP, Venugopal RB, Jabeen FTZ, Rao S. 2004. Rapid regeneration of *Mentha piperita* L. from shoot tip and nodal explants. Indian Journal of Biotechnology 3:594–598.

Gibbs PE. 1976. Studies on the breeding system of *Oxalis tuberosa* MOL. Flora 165: 129–138.

Giridhar P, Ravishankar GA. 2004. Efficient micropropagation of *Vanilla planifolia* Andr. under influence of thidiazuron, zeatin and coconut milk. Indian Journal of Biotechnology 3: 113–118.

Gonzales R, Terrazas F. 2001. Producción de papalisa y oca de calidad, para comercialización y procesamiento. Informe anual 2000–2001, Fundación PROINPA, Cochabamba, Bolivia.

Grigoriadou K, Trikka FA, Tsoktouridis G, Krigas N, Sarropoulou V, Papanastasi K, Makris AM. 2020. Micropropagation and cultivation of *Salvia sclarea* for essential oil and sclareol production in northern Greece. In Vitro Cellular & Developmental Biology–Plant 56: 1–9.

Gross R, Koch F, Malaga I, De Miranda AF, Schoeneberger H, Trugo LC. 1989. Chemical composition and protein quality of some local Andean food sources. Food Chemistry 34:25–34.

Jimenez LA. 1986. Población óptima de plantas de oca (*Oxalis tuberosa* Mol.) Anales, V Congreso Internacional de Sistemas Agropecuarios Andinos. Proyecto de Investigación de Sistemas Agropecuarios Andinos (CIID–ACDI), Puno, Peru.

Khatri P, Rana JS, Sindhu A, Jamdagni P. 2019. Effect of additives on enhanced *in vitro* shoot multiplication and their functional group identification of *Chlorophytum borivilianum* Sant. Et Fernand. SN Applied Sciences 1: 1105.

King SR. 1987. Four endemic Andean tuber crops: promising food resources for agricultural diversification. Mountain Research and Development. 43–51.

King SR, Stanley NG. 1987. Nutritional evaluation of three underexploited andean tubers: *Oxalis tuberosa* (Oxalidaceae), *Ullucus tuberosus* (Basellaceae), and *Tropaeolum tuberosum* (Tropaeolaceae). Economic Botany 41: 503–511.

Kubalakova M, Strnad M. 1992. The effects of aromatic cytokinins (populins) on micropropagation and regeneration of sugar beet *in vitro*. Biologia Plantarum 34: 578–579.

Leon J. 1964. Plantas alimenticias andinas. Bol. Tecnico Nr. 6. Instituto Interamericano de Ciencias Agrícolas, Lima, Peru.

Mejía–Muñoz JM, González–Castillo S, Mora–Aguilar R, Rodríguez–Pérez JE. 2006. Propagación *in vitro* de papa ratona (*Oxalis tuberosa* Mol.). Revista Chapingo Serie Horticultura 12: 231–237.

Morlon P, Rivera Martínez E. 2014. Comprender la agricultura campesina en los Andes Centrales: Perú–Bolivia. Institut français d'études andines, Lima, Peru.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473–497.

National Research Council. 1989. Lost crops of the Incas. National Academy Press, Washington, DC.

Ortega L. 1992. Usos y valor nutritivo de los cultivos Andinos. Programa de Investigación de Cultivos Andinos. 250–265.

Pawar BD, Jadhav AS, Kale AA, Chimote VP, Pawar SV. 2012. Zeatin induced direct *in vitro* shoot regeneration in tomato (*Solanum lycopersicum* L.). The Bioscan 7:247–250.

Pearson I. 2010. Oca's day–length thing. Available from: http://oca-testbed.blogspot.com/2010/09/ocas-day-length-thing.html (accessed November 2019).

Peixe A, Raposo A, Lourenço R, Cardoso H, Macedo E. 2007. Coconut water and BAP successfully replaced zeatin in olive (*Olea europaea* L.) micropropagation. Scientia Horticulturae 113: 1–7.

Peterson A. 2010. Growing Oca (*Oxalis tuberosa*). Available: http://chehalisvalley-altcrops.blogspot.com/2010/12/growing-oca-oxalis-tuberosa.html (accessed November 2019).

Pulgar J. 1981. Geografía del Perú – Las ocho regiones naturales del Perú. Editorial Universo, Lima.

Rivero L. 1976. Ritmo de tuberización en cinco clones seleccionados de oca. Tesis. UNSAAC, Cusco, Perú.

Rokosa MT, Danuta K. 2020. Micropropagation of *Stevia rebaudiana* plants. Ciência Rural 50: 1.

Selvakumar S, Parasurama DS. 2020. Maximization of micropropagule production in banana cultivars Grand naine (AAA) and Elakki (AB). In Vitro Cellular & Developmental Biology–Plant 56: 1–11.

Suquilanda M. 2011. Producción orgánica de cultivos andinos. Manual técnico. FAOEC, Publiasesores: Quito, Peru.

Trognitz BR, Carrión S, Hermann M. 2000. Expression of stylar incompatibility in the Andean clonal tuber crop oca (*Oxalis tuberosa* Mol., Oxalidaceae). Sexual Plant Reproduction 13: 105–111.

Trognitz BR, Hermann M, Carrión S. 1998. Germplasm conservation of oca (*Oxalis tuberosa* Mol.) through botanical seed: Seed formation under a system of polymorphic incompatibility. Euphytica 101: 133–141.

Valcárcel–Yamani B, Rondán–Sanabria GG, Finardi–Filho F. 2013. The physical, chemical and functional characterization of starches from Andean tubers: Oca (*Oxalis tuberosa* Molina), olluco (*Ullucus tuberosus* Caldas) and mashua (*Tropaeolum tuberosum* Ruiz & Pavón). Brazilian Journal of Pharmaceutical Sciences 49: 453–464.

Vuylsteker C, Dewaele E, Rambour S. 1998. Auxin induced lateral root formation in chicory. Annals of Botany 81: 449–454.

Whipkey A, Simon JE, Janick J. 1988. *In vivo* and *in vitro* lipid accumulation in *Borago officinalis* L. Journal of the American Oil Chemists' Society 65: 97.

White JW. 1975. Notes on the biology of *Oxalis tuberosa* and *Tropaeolum tuberosum*. Honors thesis, Economic Botany, Harvard College.

Yaacob JS, Yussof AIM, Taha RM, Mohajer S. 2012. Somatic embryogenesis and plant regeneration from bulb, leaf and root explants of African blue lily (*Agapanthus praecox* ssp. *minimus*). Australian Journal of Crop Science 6: 1462–1470.