

Micropropagation of *Ilex cassine* 'angustifolia'

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Abstract

For mass propagation of *Ilex cassine* 'angustifolia', tissue culture was used to shorten its propagation cycle. We sterilized its nodal segments as explants and then cultured them on MS basic medium supplemented with different concentrations of 6-BA and NAA, and placed them in the culture room for the induction and proliferation of adventitious shoots. The highest induction rate of MS medium with 6-BA 1.0 mg/L and NAA 1.0 mg/L was used for the induction of adventitious buds in *Ilex cassine* 'angustifolia' nodal segments, reaching 80%. Buds sprouted on the 10th day, while the average value of bud length reached 1.62 cm at 30 days. The adventitious buds grew fast and robust. The best proliferation coefficient was 5.7 when explants were cultured on MS basic medium containing 6-BA mg/L and NAA 0.5 mg/L.

For adventitious root induction, the adventitious shoots were transferred to MS basic medium with different concentrations of IBA and NAA. The results showed that the highest rooting rate of 98.7% was achieved when explants were cultured on MS basic medium supplemented with NAA 0.5 mg/L and IBA 1.0 mg/L, and the adventitious roots induced were thick and robust. Rooted sterile seedlings had been planted in the substrate and transferred to a greenhouse with sprinkler irrigation for further cultivation. The survival rate was about 90%.

Key message

Ilex cassine 'angustifolia' is a well-resistant holly variety with good resistance and high economic value. This research has achieved large -scale reproduction and shortening the reproductive cycle through organizational training.

Introduction

The genus *Ilex* is derived from the Aquifoliaceae, which is mostly evergreen or deciduous trees or shrubs. The genus has about 400 to 600 species, mainly distributed in tropical, subtropical to temperate regions of both hemispheres, mainly in Central and South America and tropical Asia (Chen, 1999). There are about 200 species in China, distributed on the southern slopes of the Qinling Mountains, the Yangtze River basin and a wide area to the south, and most abundant in southwest and southern China. Most of the plants in this genus are evergreen species, with graceful crowns, shiny red fruits and long-term persistence, which are good garden ornamental and urban greenery plants; flowers are many and dense, which are good nectar plants; wood is tough and meticulous, which can be used for furniture and carving; they are also rich in chemical components such as triterpenes and their glycosides, flavonoids, lignans and other chemical components, which have high medicinal value as they have the function of pain relief and swelling, heat detoxification and anti-inflammation (Zhu, 2008) such as *Ilex kudingcha* (Zuo et al., 2011), *Ilex pubescens* (Li et al., 2021) et. al, with anti-inflammatory and antioxidant, cough, pain relief, treatment of cardiovascular diseases and other effects. In South America, Paraguayan tea (*Ilex paraguariensis*) and other species are important beverages, and Dahoon (*Ilex cassine*) in southeast

America to Cuba and Bittering tea (one of them) *Ilex kudingcha* in China are also used for beverages and medicine. Therefore, plants of the *Ilex* genus have great commercial, ornamental, medicinal and health values.

Ilex cassine 'angustifolia' is a genus of *Ilex* in the Aquifoliaceae, and is one of the varieties of *Ilex cassine* (Dahon holly) cultivated in the United States. Dahon holly is native to the damp wooded vegetation of the coastal plains of the United States and usually grows in swamps and rich, moist soils; it has some salinity tolerance but is not cold hardy; in the yard, the plant is generally grown as a shrub. Compared with Dahon holly, *Ilex cassine* 'angustifolia' has excellent resistance to cold, drought and salinity, and is highly adaptable, with a beautiful shape, dense branches and foliage, colorful and richer fruit, which can enjoy the foliage and fruit, and it is an excellent greening species suitable for planting in wetlands, riverside and other areas. The plant is an evergreen shrub or small tree, up to 3–10 m in height, the whole plant is hairless, branchlets gray-brown or chestnut brown, shoots green, leaves thick papery, fully round, leaves narrowly lanceolate or linear, leaf width 1–2 cm, leaf length 5–7 cm, leaf surface green, abaxially light green, flowering in May, fruit from November to March. Although *Ilex cassine* 'angustifolia' is fast-growing and highly adaptable, due to its hard seed coat and poor water permeability, it is difficult for seeds to germinate, which is not conducive to large-scale reproduction. Since the seeds of holly have the characteristic of dormancy, which is mainly caused by the following three reasons, firstly, when the fruit of the holly plant is mature, its seed embryo is not fully developed (Tsang and Corlett, 2005); in the meantime, its seed coat, embryo and endosperm contain substances that inhibit seed germination (Liu et al., 2015); and the seed coat of the holly plant is very hard, with wax attached to the surface, and the seeds are poorly ventilated and permeable (Wang et al., 2006), which causes the seeds of holly plant are not easy to germinate.

Tissue culture uses the totipotency of plant cells, which has the advantages of high efficiency, high reproduction coefficient and the ability to maintain excellent parental traits. And we are not limited by the season when using nodal segments for tissue culture. Therefore, in this study, we screened the optimum medium for the shoot induction and proliferation of *Ilex cassine* 'angustifolia' by organogenesis, established a rapid breeding system to shorten the germination and select new cultivars efficiently and promoted the development of agriculture and forestry.

Materials and methods

A 1-year-old plant of *Ilex cassine* 'angustifolia' in the garden base of Yangtze University was used as the source of explants. Young, nonlignified branches were collected in June 2021, removed leaves, then washed with detergent to remove surface stains, then rinse with tap water for 2 h, then surface-disinfected in 3% Sodium hypochlorite solution (Tianjin Tianli Chemical Reagent Company) for 10 min, and transferred into 75% ethanol for 30 s, washed with sterile distill water for four or five times in a laminar flow hood. The branches were dissected about 1.5 cm segments of nodal with a bud. The nodal segments were transferred to culture bottle containing Murashige and Skoog (MS) basic medium (Coolaber company) plus 30 g/L sucrose and 7 g/L agar with various concentrations of plant growth

regulator for adventitious shoot induction. The pH of the media was adjusted to 5.8 with NaOH or HCL before the culture media was autoclaved at 121°C and 0.105MPa for 25 min. All bottles with explants were cultured in a growth room at a temperature of $25 \pm 1^\circ\text{C}$ with 16-h photoperiod and 2000 lux of light intensity. After 2 months, axillary shoots were excised for the following steps.

Adventitious bud induction

The sterilized nodal section (about 1.5 cm) was cultured on MS medium with different concentrations of 6-BA (0.5, 1.0, 2.0 mg/L) and NAA (0.1, 0.5, 1.0 mg/L) containing 30 g/L sucrose and 7 g/L agar at pH 5.8. A total of 10 treatments, five nodal segments were inoculated in each bottle for a total of 300 nodal segments in 60 bottles. Then explants were placed into the culture chamber at a temperature of $25 \pm 1^\circ\text{C}$ with a 16 h photoperiod and 2000 lux of light intensity. All experiments were repeated three times. After 30 days of culture, the total number of shoots, the length of shoot, the earliest initiation time, germination number, induction rate, pollution rate and induction rate were recorded. Meanwhile contaminated or browning explants were removed during culture.

Adventitious bud regeneration

For shoot regeneration, nodal segments were excised from 30-day-old fully grown in vitro shoots and cultured on MS basic medium containing with 30 g/L sucrose, 7 g/L agar, various concentrations (0.5,1.0,1.5 mg/L) 6-BA and (0.1,0.5,1.0 mg/L) NAA. The pH of the media was adjusted to 5.8 with NaOH or HCL. The explants were cultured in the culture room at $25 \pm 1^\circ\text{C}$ with a 16 h photoperiod and 2000 lux of light intensity. After 30 days, the number of shoots was recorded.

Root induction

The adventitious shoots (≈ 1.5 cm) were separated out to be cultured on the rooting culture medium. The rooting medium comprised the MS medium with 30 g/L sucrose, 7 g/L agar, NAA (0.1, 0.5, 1.0 mg/L) and IBA (0.1, 0.5, 1.0 mg/L). A total of 10 treatments, each treatment with 5 nodal segments of per culture bottle for a total of 60 bottles. All cultures were incubated in the same physical conditions as described previously. After 60 days of culture, the number of roots per explant, the length of root for each explant and root rate were recorded.

Statistical analysis

The statistical analysis of all the data was carried out using WPS (version 2020) and IBM APSS Statistical (version 24) for Variation (ANOVA) and mean separation (LSD).

Results and discussion

Shoot induction

The effect of various concentrations of plant growth regulators on shoot induction of explanted were summarized in Table 1. Shoot induction occurred to varying degrees on all test mediums (except control).

The explants did not show any bud when cultured on MS medium without phytohormone and failed to regenerate shoots after 30 days. Plant growth regulators was essential for adventitious bud induction (Thomas and Yoichiro, 2010). The concentration of 6-BA and NAA significantly affected on the length, induction rate, and the number of shoot. Our results were in general agreement with Pedro Sansberro's studies of *Ilex paraguariensis* (Sansberro et al., 1999) and Jialong Chen studies of *Ilex chinensis* (Chen, 2006). They discovered that 6-BA was a better phytohormone for shoot induction. The results showed that when the concentration of 6-BA was 1.0–2.0 mg/L, the initiation time of the adventitious bud was relatively shortened when the concentration of NAA increased. Meanwhile, the rate of the shoot and the length of shoot were significantly promoted when 6-BA 1.0 mg/L and NAA 1.0 mg/L were combined. Furthermore it was also the shortest germination days compared. the highest induction rate is 80%, and the longest bud is 1.62 cm at treatment. The number of shoots was the largest and significantly higher than other treatment (the average of 5.08 buds) when explants were grown on MS medium with 6-BA 2.0 mg/L and NAA 1.0 mg/L. Regardless of the 6-BA, the number of adventitious of the treatment with 0.1 mg/L was always less than supplemented with 0.5 mg/L or 1.0 mg/L. When 6-BA was 0.5 mg/L, the concentration of NAA had a significant effect on the number of adventitious shoots. The results showed that lower concentrations of 6-BA had no significant effect on the number of adventitious shoots. Combining higher concentration of 6-BA and lower concentration of NAA favors bud elongation. This is in agreement with the results of Sivakumar S et al. They reported that the bud growth was best in basic medium supplemented with 6-BA 1.5 mg/L and NAA 0.6 mg/L (Sivakumar et al., 2019). However adventitious bud grew slower and weak in the later stage. At the same time, explants on MS basic medium containing with lower concentration of 6-BA (0.5 mg/L) and NAA (0.1 mg/L), only a few number of buds were induction. This indicates that lower concentration of 6-BA had less effectiveness for shoots induction and higher concentration of 6-BA significantly inhibited the growth of the shoot too. The results were still supported by those reported for *Ilex glabra* (Li, 2014), *Ilex kudingcha* (Yao et al., 1996) and *Ilex paraguariensis* (Ana et al., 2014), which also indicated that higher or lower concentrations of 6-BA are inhibitor of shoots growth. During the adventitious bud induction and proliferation phase, 6-BA plays an important role, because the chemical stability of BA is the highest among the group of cytokinins in vitro plant, and the quantity of BA declined in the medium is the lower rate (Klemš et al., 2000 ; Rahman et al., 2006).

Table 1

The influence of different concentrations of plant growth regulator on shoot induction of *Ilex cassine* 'angustifolia'

Plant growth regulators		induction rate(%)	Pollution rate(%)	the earliest initiation time	Average no.of shoots	Length of shoots(cm)
6-BA (mg/L)	NAA (mg/L)					
0.5	0.1	20.00i	0c	15	2.09 ± 0.30fg	1.26 ± 0.28def
0.5	0.5	46.67h	20.00a	21	2.42 ± 0.52defg	1.57 ± 0.41abc
0.5	1.0	60.00d	0c	20	2.13 ± 0.35fg	1.34 ± 0.33bcd
1.0	0.1	40.00i	0c	18	2.09 ± 0.30fg	1.08 ± 0.33f
1.0	0.5	73.33b	6.67b	13	3.67 ± 0.78bc	1.33 ± 0.33cde
1.0	1.0	80.00a	0c	10	4.38 ± 0.77ab	1.62 ± 0.41a
2.0	0.1	66.67c	20.00a	18	3.30 ± 0.95cd	1.10 ± 0.31ef
2.0	0.5	66.67c	0c	15	3.90 ± 1.10bc	0.79 ± 0.22g
2.0	1.0	53.33e	0c	13	5.08 ± 1.31a	0.67 ± 0.19g
0	0	0j	0c	/	0h	0h
Different letters in the same column indicate a significant difference in the levels between the two treatments, $p < 0.05$; and the same letters indicate an insignificant difference						

Shoot proliferation

The plant growth regulator concentrations had a significant effect on shoot proliferation. Explants were transferred to proliferation medium and recorded the growth situation after 30 days. Explants culture on MS medium without cytokinin or auxin yielded no proliferation. We found that 6-BA could better promote the bud differentiation in the proliferation stage. The number of buds per explants on MS medium plus 6-BA 1.5 mg/L and NAA 0.5 mg/L was significantly higher than other treatments (Table 2). This indicates facilitating the formation of adventitious shoots at large ratios of cytokinin and auxin, but the overall concentration should not be too high. These results were still supported by those reported for *Ilex latifolia* (Liang et al., 2003) and *Ilex verticillata* A. Gray (Sun et al., 2010) and *Ilex dumosa* (Luna et al., 2003), which also concluded that the appropriate concentration of 6-BA could promote bud proliferation in the shoot regeneration stage. Regardless of the 6-BA concentration, the proliferation coefficient in the treatment containing 1.0 mg/L NAA were always lower than 0.1 mg/L or 0.5 mg/L. The results showed that higher concentrations of NAA could inhibit the number of buds per shoot. The results obtained confirm, in agreement with another report that higher concentration of NAA resulted in complete inhibition of bud formation even when combined with 6-BA for *Ilex dumosa* (Luna et al., 2003). However, when the

concentration of 1.0 mg/L, the proliferation coefficient of the containing 1.0 mg/L NAA was not significantly difference from the containing 0.5 mg/L NAA. Meanwhile, MS basic medium supplemented with 6-BA 1.5 mg/L and NAA1.0 mg/L had in fewer shoots as compared to other concentration of 6-BA and NAA. The result was in agreement with the previous report on the *Ilex crenata* (Zhu, 2006) and *Gloriosa superba* (Kahate, 2017), which could inhibit shoot proliferation when higher concentrations of 6-BA and NAA were used simultaneously. In summary, the largest proliferation coefficient (5.4 ± 0.3) was recorded when the concentration of 6-BA was 1.5 mg/L and NAA was 0.5 mg/L.

Table 2
The Influence of different concentrations of 6-BA and NAA on shoot regeneration of *Ilex cassine* 'angustifolia'

Plant growth regulators		New shoots
6-BA (mg/L)	NAA (mg/L)	
0.5	0.1	$4.8 \pm 0.5b$
0.5	0.5	$4.3 \pm 0.2b$
0.5	1.0	$3.2 \pm 0.4c$
1.0	0.1	$4.2 \pm 0.2b$
1.0	0.5	$3.3 \pm 0.2c$
1.0	1.0	$3.2 \pm 0.2c$
1.5	0.1	$4.3 \pm 0.7b$
1.5	0.5	$5.7 \pm 0.3a$
1.5	1.0	$2.7 \pm 0.1c$
0	0	0
Different letters in the same column indicate a significant difference in the levels between the two treatments, $p < 0.05$; and the same letters indicate an insignificant difference		

Roots induction

All treatments had various degrees of rooting induction, except that explants did not induce roots when on MS medium with phytohormone-free. The percentage of rooted explants ranged from 37.8–98.7% (Table 3). Thus both NAA and IBA has a significant impact on the rooting of *Ilex cassine* 'angustifolia'. We found that the highest rooting rate at 98.7% was cultured on MS basic medium with NAA at 0.5 mg/L and IBA at 1.0 mg/L after 60d of culture. It showed that low concentrations of NAA combined with high concentrations of IBA can induce strong taproots and fibrous roots. The results were in agreement with Qun Shi (Qun, 2017), he found that roots growth was best achieved when explants were cultured on MS medium with IBA and NAA at the concentration of 1.0 mg/L and 0.2 mg/L. The highest value of root length (2.6 cm) was obtained when shoots were cultured on MS medium containing NAA 1.0 mg/L

together with IBA 0.1 mg/L which was significantly longer than all other treatments. However the induction roots were not only lanky but also weak. The number of the roots was the peak at 3.7, when explants were cultured on MS medium supplemented with NAA 0.1 mg/L and IBA 0.1 mg/L. We observed that lower concentration of IBA could cause weak and slender roots and higher concentration of IBA would promote root growth. The result was in general agreement with previous reports for the study of *Petrocosmea grandifolia*. Their studies found that IBA can effectively improve the rooting rate (Yang et al., 2019). Higher concentration of NAA could decrease the rooting rate but shortened the rooting time, when explants were cultured on MS medium supplemented with NAA and IBA. The result was in general agreement with previous reports for the study of *Ilex pubescenas* (Liu et al., 2014). Although only different basic medium was used, they reported that explants had robust roots and grew well when they were grown on 1/2 MS medium supplement NAA at 0.2 mg/L and IBA 0.1 mg/L. This also proves that the combination of IBA and NAA has great results on induced rooting. On the other hand, in the study by Horbach (Horbach et al., 2011) and Zhijian Zhou (Zhou et al., 1996), higher concentration IBA could inhibit rooting. In our study, IBA can effectively thicken the root synodal, which may be due to different plants, contrary to the conclusion of Yang Hongqiang et. al (Yang et al., 2012).

Table 3
The Effect of different concentration of plant growth regulator on root formation of *Ilex cassine* 'angustifolia'

Plant growth regulators		Rooting(%)	Average no.of roots per shoot	Average root length(cm)
6-BA (mg/L)	NAA (mg/L)			
1	0.1	0.1	92.8c	3.7a
2	0.1	0.5	38.5g	2.7c
3	0.1	1.0	94.7b	2.1de
4	0.5	0.1	74.3d	2.4cd
5	0.5	0.5	95.3b	2.2de
6	0.5	1.0	98.7a	3.2b
7	1.0	0.1	63.6e	1.8e
8	1.0	0.5	58.4f	3.1b
9	1.0	1.0	37.8g	1.4f
Different letters in the same column indicate a significant difference in the levels between the two treatments, $p < 0.05$; and the same letters indicate an insignificant difference				

The in vitro-derived plantlets with well-developed roots were removed from the medium after 60 days of culture and treated with fungicide and washed in sterile distilled water. The plantlets rooted were successfully acclimatized in mixture substrate under greenhouse conditions. Transplanted into the

cultivation substrate and grown under the spray synodal in the greenhouse seedbed, the survival rate of transplants is about 90%.

In this experiment, the explants of *Ilex cassine* 'angustifolia' did not appear vitrification, which is not consistent with the slightly serious vitrification of the explant material in other reports on *Ilex verticillata* (L.) A. Gray 'Oosterwijk' (Zhang et al., 2014; Lei and Wu, 2019), considering whether it is a genetic factor or the cause of endogenous hormones. In the future, its adaptability to different environments is the direction of further research.

Conclusion

In this study, the nodal segments of *Ilex cassine* 'angustifolia' was used as the explant material for adventitious bud induction, the highest induction rate was 80% when the explants were cultured on the MS basic medium supplemented with 1.0 mg/L of 6-BA and 1.0 mg/L of NAA, the explant sprouts were observed on the tenth day. The average length of peer shoot was 1.62 cm after 30 days, adventitious buds grow fast and strong. Secondary proliferation stage, terminal shoots were cultured on MS basic medium with 6-BA 1.5 mg/L and NAA 0.5 mg/L, the highest proliferation coefficient was 5.7. The explants transferred from the proliferation culture media to the rooting media. MS medium with NAA 0.5 mg/L and IBA 1.0 mg/L as the optimal rooting culture medium, the highest rooting rate at 98.7%, and the induced adventitious roots were thick and robust. Then transplanted the plantlets into the mixture of perlite and peat (1:1 by volume) and acclimated under the mist benches in a greenhouse, when the explants in vitro have produced roots. The survival rate is 90% at this time.

Abbreviations

6- BA
7- 6-benzyaminopurine
NAA
Naphthalene acetic acid
IBA
indole-3-butyric acid
MS
Murashige and Skoog

Declarations

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Data Availability

All data generated or analysed during this study are included in this published article

Ethics declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

Conflict of interest The authors declare no conflict of interest exists.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

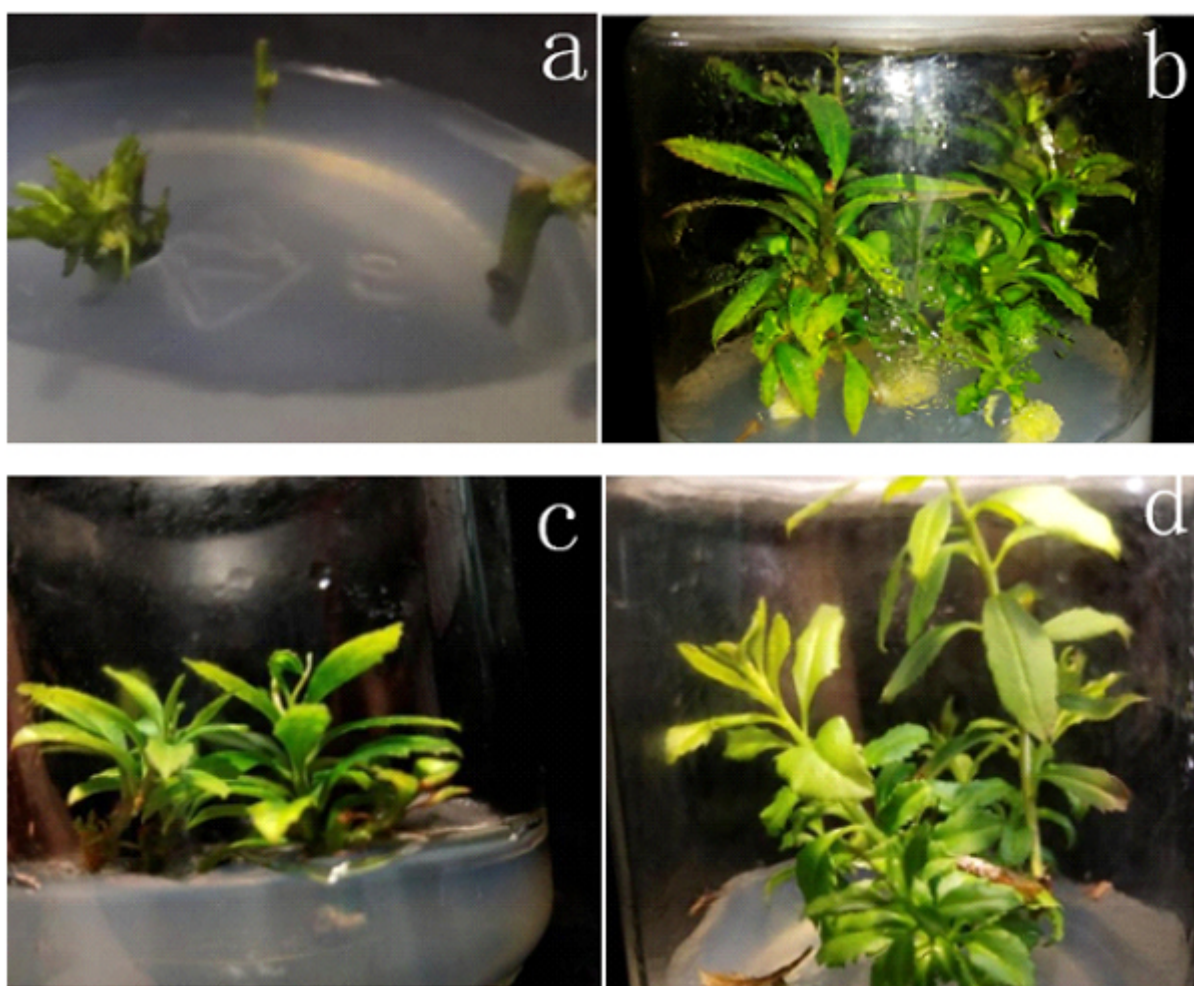


Figure 1

Growth of *Ilex cassine* 'angustifolia' adventitious buds under different treatments (a).explants germination on MS with 6-BA 1.0 mg/L and NAA 1.0 mg/L after 10 days (b). the highest induction rate (c).explants germination on MS with 6-BA 2.0 mg/L and NAA 1.0 mg/L (d).explants germination on MS with 6-BA 1.0 mg/L and NAA 1.0 mg/L after 30 days

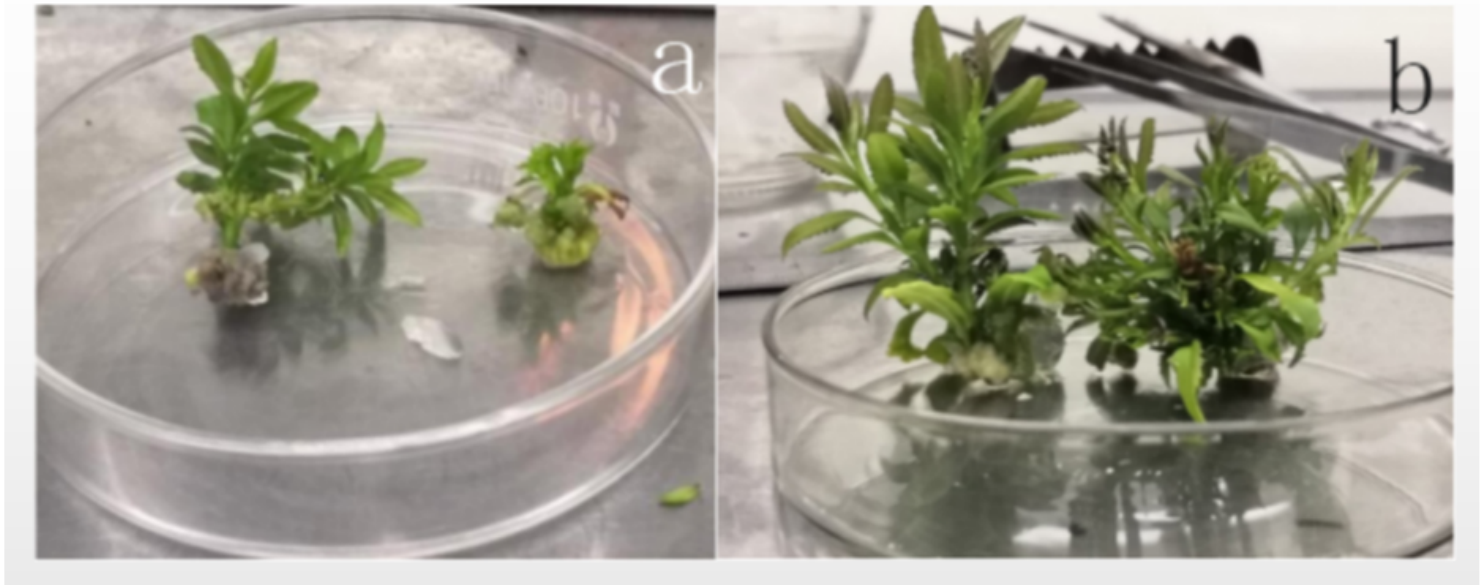


Figure 2

The proliferation of *Ilex cassine* 'angustifolia' adventitious shoots under different treatments (a).explants germination on MS plus 6-BA 1.5 mg/L and NAA 1.0 mg/L (b).explants germination on MS plus 6-BA 1.5 mg/L and NAA 0.5 mg/L



Figure 3

The morphology of *Ilex cassine* 'angustifolia'adventitious roots under different treatments (a).roots culture on MS plus IBA 1.0 mg/L and NAA 0.5 mg/L (b).roots culture on MS supplemented IBA 0.1 mg/L and NAA 1.0 mg/L (c).roots culture on MS plus IBA 0.1 mg/L and NAA 0.1 mg/L