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AIR-LAYERING TECHNIQUE IN SWEET ORANGE (*Citrus sinensis* (L.) Osbeck) USING AUXIN AND STEROIDAL EXTRACT ON COCONUT HUSK (COCOPEAT)

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ABSTRACT

Air layering is a propagation technique used to produce clones of woody plants that are difficult to root from cuttings; it induces root formation on branches while they remain attached to the parent plant. In this study, coconut husk (cocopeat) was used as the rooting medium for air-layering Citrus sinensis (L.) Osbeck (sweet orange). The aim was to propagate new trees from the stems of a single parent plant as a conservation technique. A young, straight, healthy, and vigorous stem was selected; side branches and leaves were removed from a 10–15 cm section. A 2-cm-wide girdling cut was made around the stem through the bark and cambium. Approximately 30g of coconut husk was packed around the cut inside a polythene bag, which was then loosely wrapped with twine or plastic tape and sealed at one end with waterproof adhesive tape to produce a packed medium 5–7 cm thick. Auxin (AP) and steroidal extracts (SP) were inoculated into the cocopeat at concentrations of 100, 200, and 300 mg/mL. The experiment ran for 3 months; after initial rooting, root number, root length, and root circumference were assessed for 20 days. Differences among treatment groups were statistically significant for all parameters ($P < 0.001$). Steroidal extracts (SP at 100, 200, and 300 mg/mL) produced the highest root numbers in a dose-dependent manner with an initial rooting period of 45 days, suggesting that steroidal extract is an effective rooting enhancer with potential to replace auxin in plant propagation.

Keywords: Air-layering, *Citrus sinensis,* Auxin, Steroidal Extract, Callus, Vegetative propagation

Introduction

Citrus sinensis (L.) Osbeck, commonly known as sweet orange, is one of the most economically important fruit crops in the world. It belongs to the family Rutaceae and is widely cultivated in tropical and subtropical regions for its nutritious fruits, which are rich in vitamin C, antioxidants, minerals, and dietary fiber. Sweet orange contributes significantly to food security, income generation, and the fruit-processing industry. In Nigeria and many other developing countries, citrus production plays an important role in rural livelihoods and agricultural development.

The propagation of citrus is traditionally achieved through seeds, budding, and grafting. However, seed propagation often results in genetic variability and prolonged juvenile periods before fruiting, making it unsuitable for maintaining desirable cultivar characteristics. Vegetative propagation methods are therefore preferred because they produce true-to-type plants that retain the genetic traits of the parent plant (Rafieq *et al.,* 2016; Leakey, 2017). Air-layering, also known as marcotting, is one such vegetative propagation technique that induces root formation on a stem while it remains attached to the parent plant. Once sufficient roots have developed, the rooted branch is detached and established as an independent plant. This method allows the propagule to continue receiving water and nutrients from the mother plant during root development, thereby increasing the chances of successful establishment (Wang *et al.,* 2005).

Auxin is a plant hormone that regulates the development of the root and can also stimulate xylem tissue differentiation. It is an organic substance that promotes the growth and development of plants at low concentrations. The maximum number of primary roots developed per rooted layer on air and ground layers were recorded with the application of IBA + NAA at 6000 ppm (Chatterjiee *et al*., 1989). Auxins regulate numerous developmental processes in plants, including cell expansion, root initiation, vascular tissue differentiation, bud and flower growth (Wang *et al.,* 2005). Auxin and cytokinin are the main phytohormones that control root growth, root gravitropism, and vascular differentiation. The characterization of auxins as root-developing hormones in plants established a link between these molecules and root development. Since auxins were first described, there has been a tight connection between this class of hormones and root development (Tatematsu *et al.,* 2004; Evans *et al*., 2009). Auxin can be synthesized in young leaves and cotyledons (Tromas *et al.,* 2009). Most forbs in the families Cucurbitaceae, Vitaceae, and Rubiaceae contain considerable amounts of steroids, which are growth enhancers (Amodu *et al*., 2020).

Plants that need greater efficiency in water use have a strategic demand to retain higher carbon dioxide (CO_2) concentrations inside. Characteristics of leaf anatomy related to water deficit are reduction of thickness, higher cell density, and smaller intercellular spaces, all of which try to mitigate the problems of excessive water loss (Chartzoulakis *et al*., 2002; Evans *et al*., 2009). The relationship between the root and the shoot is strongly modified due to conditions of water availability. One way that plants keep their water status stable is by reducing the growth of the shoot, reduce their leaf area and water loss to the atmosphere (Evans *et al*., 2009).

Water is essential for successful air-layering because it maintains adequate moisture around the girdled stem, promotes callus formation, and stimulates the initiation and development of adventitious roots. A continuously moist rooting medium prevents desiccation of the wounded tissues and facilitates the accumulation and translocation of carbohydrates, auxins, and other rooting cofactors required for root development. Consequently, higher rooting success is generally obtained when sufficient moisture is maintained around the layered portion of the stem (Hartmann *et al*., 2011; Bareja, 2021). Furthermore, the use of moist substrates such as sphagnum moss provides the moisture and aeration necessary for root initiation and growth, while dry conditions

can inhibit root formation. Conversely, excessive moisture may reduce oxygen availability, leading to poor root development and tissue decay (Bareja, 2021).

Anatomically, the change in vascular diameter may be a response to water deficit conditions and tends to decrease under water deficit (Kutlu *et al*., 2009). Under conditions of potassium deficit, sugars from starch hydrolysis become more relevant in osmotic regulation than in the process of stomatal opening. The proline amino acid also stands out as an osmotic regulator and is linked to stress both by water deficit and salinity (Molinari *et al*., 2004). Nevertheless, proline does not only exert the function of osmoregulation in plant cells during periods of water deficit, but it can also protect against the activity of free radicals, regulate the pH in the cytoplasm, protect against the denaturation of macromolecules and act as a source of carbon and nitrogen under conditions of stress (Vanrensburg *et al*., 1993).

Coconut coir is the fibrous material found between the hard, internal shell and the outer coat of a ripe coconut. It is a 100% renewable resource that is odorless, easy to handle, and uniform in composition. It promotes strong root growth and plant vigor and has an ideal pH range of 6.0-6.8, it has high water retentive capacity, and it contains significant amounts of phosphorus and potassium. It is used as a growing medium for many crops such as fruit trees, vegetables, and cut flowers (Gunasekaran and Poornima, 2018). Coconut coir also retains plant nutrients, reduces leaching of substances, and shows response to excipients when used as an adjuvant growth medium, revealing vigor and other important characteristics of plantlets (Thomas *et al.,* 2013; Thompson *et al.,* 2009).

The objective of the study was:

1. To create clones of plants with interesting characteristics using sweet orange as a model
2. To examine the combined effect of growth hormones (auxin and steroidal extract) in Cocopeat on the plant.
3. To establish a sustainable field template for conservation of rare and threatened species.

Anthropogenic activities, climate change, pest & diseases and ecological drift has led to mass extinction of some plant species and the red lists are on the increase. One of the aforementioned factors was also the reason for decrease in the population of sweet orange in federal University of Lafia botanical garden. For these reasons, there is an urgent need to use and document air-layering technique as an asexual means of propagation that helps to produce complete replica of a plant within few months. And as a conservation technique, air-layering help to fight species extinction by producing multiple replica based on the number of branches available on the plant.

Materials and Methods

Experimental location

The field work was carried out at the Botanical Garden of the Department of Plant Science and Biotechnology, Federal University of Lafia (FULafia), Nasarawa state. The study area falls within the guinea savannah zone of North Central Nigeria, and is located between latitude 08.5060ºN and longitude 08.5227ºE.

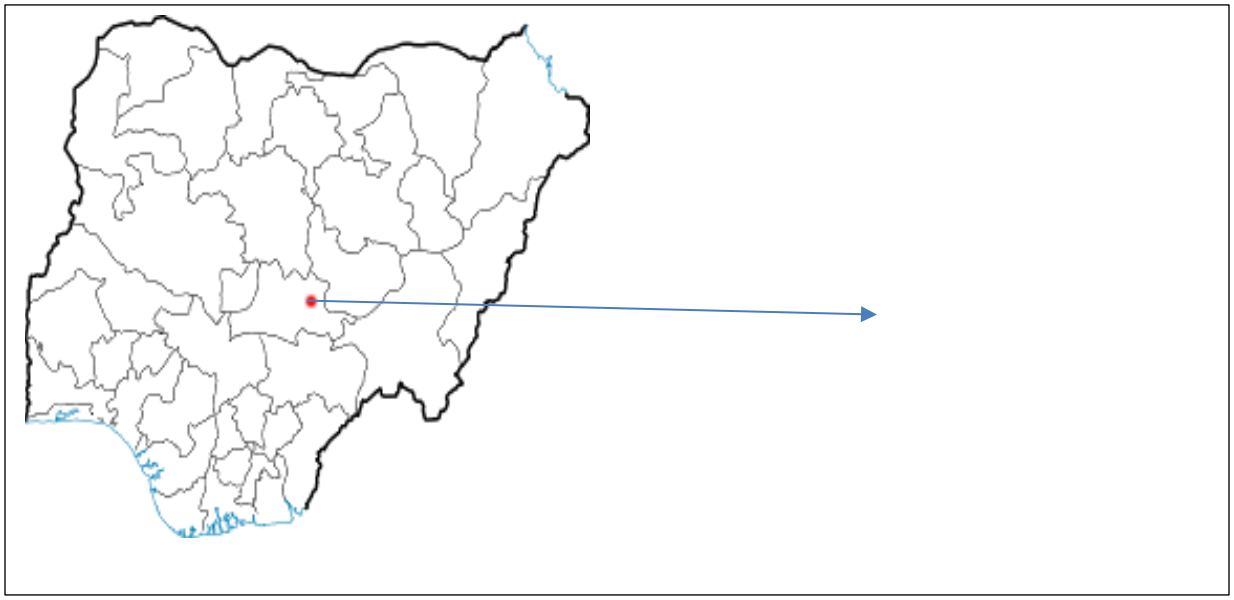


Fig. 1. Experimental location (Google mobile map)

Experimental design and procedure

The experiment was arranged in a Randomized Complete Block Design (RCBD) with ten blocks (replicates). Each block contained the seven treatments: AP1, AP2, AP3, SP1, SP2, SP3, and the control. Within each block, treatments were randomly assigned to experimental units, ensuring that every treatment appeared once in each block. The blocking was employed to minimize the effects of environmental heterogeneity across the experimental area, thereby improving the precision of treatment comparisons. A one- to two-year-old stem of sweet orange that is straight, healthy, and vigorous was selected, and the side branches and leaves were trimmed off from about 10 cm to 15 cm sections of the internodes. The stem was incised round, making a 2 cm cut through the internode in a circumference. About 30 g of coconut husk was packed around the cut in a polythene bag. The cut stem section was wrapped loosely with twine or plastic tape and sealed at one end with weather-proof adhesive tape. The wrapped coconut husks make a thickness of 5-7cm around the stem. The treatments were applied at different concentrations (100 mg/mL, 200 mg/mL, and 300 mg/mL) for auxin and steroidal extract (AP1, AP2, AP3 and SP1, SP2, SP3), respectively, on an interval of 3 days using a 5cm syringe pierced through the packed coconut husk. The polyethene were opened and checked occasionally for signs of rooting at an interval of two days (David, 2002; Alex, 2015).

Data collection

Rooting was observed few days after callus formation, the results were collected and tabulated under the following rooting characteristics such as root length, root circumference and root numbers. Other parameters considered were, days of callus formation and days of rooting. The root characters were measured from the day of rooting to 20 days after, using thread and meter rule.

Statistical analysis

The descriptive statistics as table and graphs. Analysis of variance (one-way) was used to evaluate the role of the treatments on the root number, root length and root circumference. This was followed by *post hoc* test, least significant difference (LSD) for pairwise multiple comparison

to determine which treatments were responsible for the observed differences. Chi-square tests were used to compare number of days of callus formation and rooting across the groups. Normality of distribution of continuous variables was analyzed using the Shapiro-Wilk test. All tests were two-tailed with a *P-value* <0.05 as the limit of statistical significance.

Results

The results revealed successful callus and root formation across all treatments, including the control. However, treatments receiving the steroidal extract exhibited superior rooting performance compared to the auxin-treated and control groups. Root number increased progressively with increasing concentrations of the steroidal extract, indicating a dose-dependent response. The highest root production was observed in SP3 (300 mg/ml), suggesting that the steroidal extract effectively enhanced adventitious root initiation and development. These findings demonstrate the potential of the steroidal extract as a rooting promoter in air-layering, with efficacy increasing as the concentration of the extract increased (see table 1).

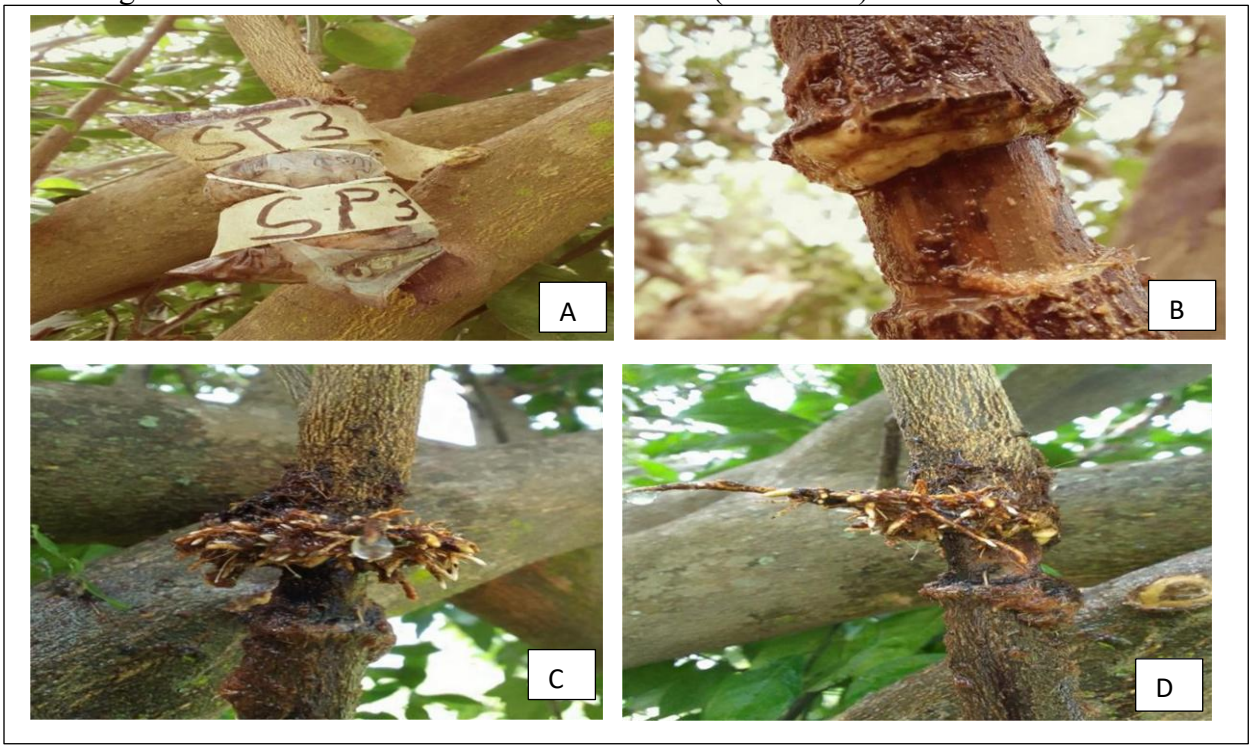


Fig. 2: Air-Layered *Citrus sinensis*

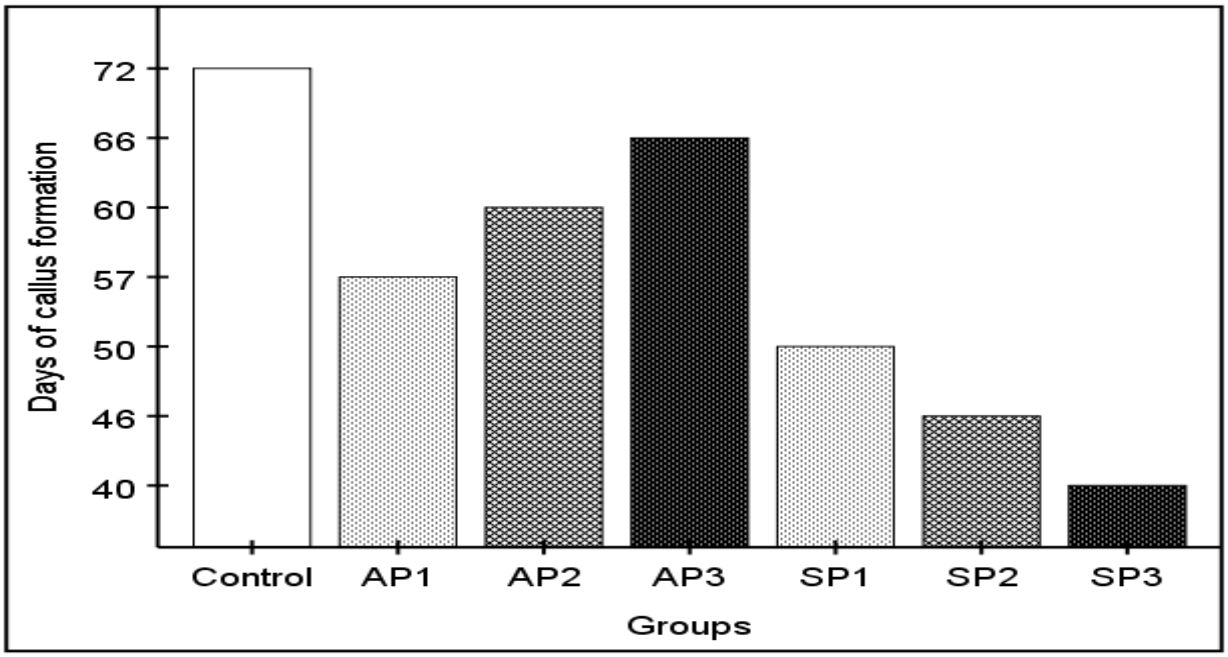
Key: **A** (Experimental label), **B** (callus formation), **C** (initial root formation), **D** (subsequent root formation).

Table 1: Effect of Different Concentration of Steroidal Extract and Auxin on Root Growth of Sweet Orange.

TREATMENTS	ROOT NUMBER Mean ± SD	ROOT LENGTH Mean ± SD	ROOT CIRCUMFERENCE Mean ± SD
AP1 (100 mg/ml)	37.00 ± 0.94*	2.85 ± 0.30*	0.76 ± 0.21*
AP2 (200mg/ml)	34.50 ± 1.08*	3.53 ± 0.22*	0.50 ± 0.19
AP3 (300mg/ml)	39.30 ± 1.06*	3.34 ± 0.23*	1.0 ± 0.26*
SP1 (100mg/ml)	45.10 ± 1.45*	4.00 ± 0.31*	0.59 ± 0.15
SP2 (200mg/ml)	51.10 ± 1.45*	4.25 ± 0.30*	0.74 ± 0.11*
SP3 (300mg/ml)	60.20 ± 1.48*	4.82 ± 0.35*	1.09 ± 0.27*
CONTROL	32.50 ± 0.85	2.16 ± 0.28	0.53 ± 0.18
P-VALUE	<0.001	<0.001	<0.001
LSD	1.083	0.259	0.185

Note: The treatment group whose mean difference to the control is greater than or equal to the LSD is considered significant and indicated with an asterisk.

All the measured outcomes (root number, root length, and root circumference) show highly significant differences among treatments overall ($p < 0.001$). Most treatments significantly increased root number and root length compared to the control. The SP series shows a clear dose-response: SP1 < SP2 < SP3 in all three measures. Root circumference increases are significant for AP1, AP3, SP2, and SP3, but not for AP2 or SP1. The largest effects are from SP3 (300 mg/mL): more roots, longer roots, and larger circumference compared to the control. SDs are relatively small compared with means, so group means appear precise.



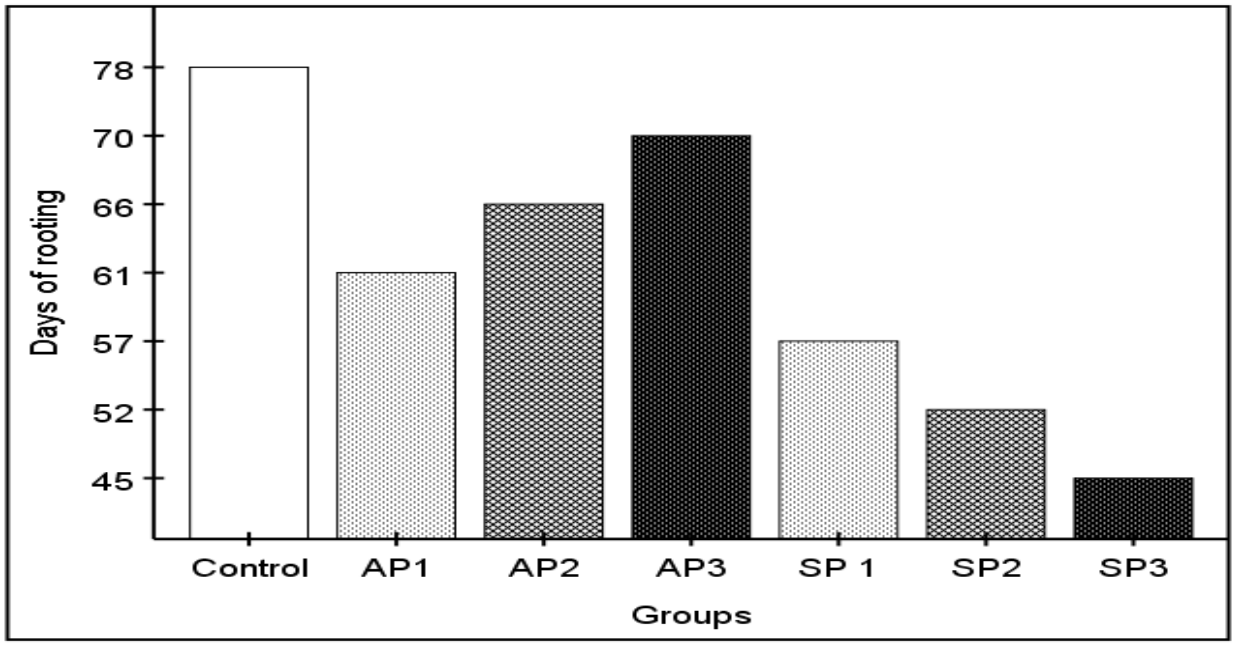
$\chi^2 = 13.69$, $P = 0.033$.

AP= auxin, SP= steroidal extract (AP1= 100 mg/mL, AP2= 200 mg/mL, AP3= 300 mg/mL and SP1= 100 mg/mL, SP2= 200 mg/mL, SP3= 300 mg/mL)

Figure 3: Days of callus formation of *Citrus sinensis* as exposed to auxin and steroidal extract

There was a significant difference in days to callus formation between the Auxin (AP) and steroidal extract (SP) treatments ($\chi 2 = 13.69$, $P = 0.033$).

The number of days required for callus formation decreased with increasing concentrations of the steroidal extract, indicating a dose-dependent response. Among the treatments, SP3 (300 mg/mL) exhibited the fastest callus formation, producing callus in less than 40 days. In contrast, the control treatment required the longest period for callus initiation, taking 72 days. AP3 (300 mg/mL) recorded the longest callus formation period among the auxin treatments, taking approximately 66 days.



$\chi^2 = 12.20$, $P = 0.058$

AP= auxin, SP= steroidal extract (AP1= 100 mg/mL, AP2= 200 mg/mL, AP3= 300 mg/mL and SP1= 100 mg/mL, SP2= 200 mg/mL, SP3= 300 mg/mL)

Figure 4: Days of root formation of *Citrus sinensis* as exposed to auxin and steroidal extract.

There was a trend toward a difference in days to root formation between treatments, but it did not reach statistical significance at α = 0.05.

The number of days required for root initiation decreased with increasing concentrations of the steroidal extract, indicating a dose-dependent effect. Among the treatments, SP3 (300 mg/mL) recorded the shortest rooting period, with root emergence occurring after 45 days. In contrast, the control treatment required the longest time for root initiation, taking 78 days. This result suggests that the steroidal extract accelerated adventitious root formation and enhanced rooting efficiency. Conversely, the auxin-treated specimens exhibited an opposite trend, as the time required for root initiation increased with increasing auxin concentration. AP3 (300 mg/mL) recorded the longest rooting period (70 days) among the auxin treatments, indicating that higher auxin concentrations may have inhibited or delayed root initiation in the air-layered propagules.

Discussion

The results were virtually significant for the whole treatments but yielded a better result for root numbers in a dose dependent manner for steroidal extract in SP1, SP2, and SP3 (see table1), but fluctuate in auxin treatments, with AP2 lower than AP1 and AP3, this partially conform with the study of Barbour *et al.* 1998 which state that in roots, the most well-characterized auxin-associated phenotypes are the dose-dependent, increase in the length of epidermal-derived root hairs, the bimodal effect of auxin concentration on primary root length, the dose-dependent

increase in number of lateral root primordial, and the response to gravity. The control also shows a positive response to rooting (see fig. 4), which disagrees with the study of Ibukun (2016) on *Blighia sapida* where no rooting was observed within the untreated stem.

The rapid callus formation observed in the steroidal extract treatments, particularly SP3, demonstrates the effectiveness of the extract in stimulating cell division and tissue proliferation in a dose-dependent manner. The reduction in the number of days required for callus initiation with increasing extract concentration suggests that the steroidal compounds enhanced the physiological processes involved in wound healing and callogenesis. In contrast, the prolonged period required for callus formation in the auxin-treated specimens, especially AP3, indicates that increasing auxin concentration may have slowed the callogenesis process. Similar studies have reported that while auxins promote callus formation, excessive concentrations can disrupt normal cellular differentiation and delay tissue development (Hartmann et al., 2011; Taiz et al., 2015).

The reduced rooting period observed with increasing concentrations of the steroidal extract suggests that the extract promoted rapid cell differentiation and adventitious root initiation in a dose-dependent manner. The shortest rooting time recorded in SP3 (45 days) indicates that higher concentrations of the steroidal extract enhanced rooting activity compared with the control, which required 78 days to produce roots. In contrast, the increase in rooting time with increasing concentrations of auxin suggests that the applied concentrations may have exceeded the optimum level required for rooting. Excessive auxin concentrations have been reported to inhibit root initiation and elongation, thereby delaying root development (Hartmann *et al*., 2011; Davies, 2010). These findings indicate that the steroidal extract was more effective than the auxin treatment in reducing the time required for root formation during air-layering.

SP3 shows a significant difference across rows with the best yield in a steroidal dose of about 300mg/ml, which promotes auxin accumulation in roots, in response to residual sucrose from the substrate, thereby regulating root growth, which agrees with Wang *et al*. (2005). In fig. 2 (Plates C & D), the result is significant (P <0.05) for the days of callus formation, which can be seen in all the groups, especially SP3. It was also observed that in days of callus formation and days of rooting, auxin and steroidal treatment were dose-dependent in a reverse manner (see fig. 3 & 4), i.e., the lower the dose, the faster it forms callus and roots in auxin treatment. The higher the dose, the faster it forms callus and root in steroidal treatment.

Conclusion

Steroidal extract aids root formation faster than auxin and the standard (control), as seen in AP1, AP2, AP3, SP1, SP2, and SP3. The aforementioned method is vital for species conservation as substrate inoculation yielded better results against the standard by recording zero mortality due to the Water retention, aeration, nutrient availability, and disease resistance of the cocopeat and extract functioning in synergy. From the above studies, it is concluded that Steroidal Extract aided root formation faster than auxin and the control treatment, as seen in SP3 (300 mg/ml)

Recommendations

In line with the findings, the following are recommended:

1. The air-layering technique should be encouraged to prevent loss of plants (trees) from the ecosystem.
2. Awareness should be created among researchers and farmers to use air-layering techniques because it is cheap and simple to learn.
3. Steroidal extracts should be used because of their rapidity in root formation. It is recommended that farmers and nursery operators adopt the use of steroidal extract at a concentration of 300 mg/mL (SP3) for the vegetative propagation of plants through stem cuttings and air-layering. The treatment enhanced root initiation more rapidly than both

auxin and the controls, suggesting its potential to improve propagation efficiency, increase the establishment rate of air-layering, and shorten nursery production time. The use of steroidal extract may therefore serve as an effective alternative to conventional rooting hormones, particularly where rapid root development is desired. However, farmers should apply the extract at the recommended concentration and under appropriate nursery management conditions to achieve optimal results

Conflict of Interest

The authors declare that there is no conflict of interest.

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