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Influence of mercuric chloride and sodium hypochlorite on apical and axillary buds regeneration of *Colocasia esculenta* in tissue culture

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ABSTRACT

Taro (*Colocasia esculenta* L. SCHOTT) is a staple food in many southern countries and an ancient starchy crop consumed by more than 400 million people. It is treated by diseases and pests that affect seed availability. Thus, the techniques of in vitro culture mostly used to overcome the problem of seeds production meet enormous difficulties of infection and necrosis of the explants. This study aims to determine the optimal use of mercuric chloride and sodium hypochlorite for disinfection of apical and axillary buds of taro. For this purpose, three doses of sodium hypochlorite (8%, 10% and 12%) and mercuric chloride (0.08%, 0.1% and 0.15%) were used with three immersion times (25 min, 30 min and 45 min) for sodium hypochlorite and (5min, 7min and 10min) for mercuric chloride. A binary logistic analysis was performed to understand or predict the effect of different doses of NaOCl and HgCl2 on the behavior of apical and axillary buds of taro. The results showed that 8% sodium hypochlorite with immersion time of 25 minutes is favorable for the disinfection of both apical and axillary explants of taro. For mercuric chloride, only the dose of 0.15% is effective for apical bud survival. The present study offers an opportunity to make available the seed of taro through the organogenesis of the species without any risk of infection.

Keywords: *Colocasia esculenta*, *in vitro*, desinfection.

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Introduction

Taro (*Colocasia esculenta* L. SCHOTT, 1832) is a monocotyledonous plant of the family of *Araceae*. It is a starch crop consumed by more than 400 million people and constituted fifth among the tubers crops after [1]. In the Pacific, where taro is highly consumed and played an importance role in the diet of the populations, it provides between 15 and 53% of the food energy to consumers [2]. It contains high carbohydrate (21% in the fresh matter) that makes it an energy starter of choice [3] which is superior to other tuber and root crops. Its digestibility is estimated at 98.8%. Taro's tubers are very digestive and also a good source of calcium and iron [4]. In addition, taro provides very broad leaves (8 to 25 cm) that are used as vegetables in culinary preparations [5]. The leaves provide a large amount of vitamin A, which is necessary for good growth, eye health and disease prevention. They also contain vitamin C and vitamin B2 (riboflavin) [6]. Despite the nutritional importance of taro and its role in food security, the crop is confronted to many disease and pests reducing highly the yield [7]. In addition, the tuber (corme) has a high water content (75%) makes it no easier for conservation as seed [8]. Therefore, the tubers or infected were mostly used to establish a new plantings. This favors a high dissemination of pathogens in the growing areas. As a result, yield decreases and crop quality deteriorates. According to [9] and [10], pathogens such as *Pythium myriotylum* and the taro mosaic virus (TMV) can cause a 50-90% loss of production. Furthermore, a much part of yield is used as seed to cover the production of next season. This traditional practice is common to many root and tuber crops excluded farmers of a substantial amount of consumable tubers [11]. The deficit of healthy seeds then appears as one of the major constraints to taro production. Thus, it is essential to look for biotechnology tools through micropropagation in vitro to regenerate a hole plant and identical to the parent plant. This will ensure a permanent availability of healthy seeds. However, the success of in vitro micropropagation depends on several factors including the disinfection of the explant. For this purpose, mercuric chloride has been identified as an effective disinfectant on taro explants before in vitro culture [12, 13 and 14]. Its efficacy has also been demonstrated in apical buds of taro [15 and 16]. For the same purpose, [17, 11 and 18] used sodium hypochlorite for the disinfection of the apical and axillary buds of the species. These authors neither evaluated the immersion time of the explants nor varied the concentrations of the disinfectants; factors influencing the survival of explants in vitro [19 and 20]. Based on these results, this study aims to identify the optimal conditions for disinfection of taro in Benin. It is based specifically on the evaluation of the effect of various concentrations of mercuric chloride, sodium hypochlorite and immersion time on the survival of apical and axillary buds of taro.

Material and Methods

The corms of taro (*Colocasia esculenta*) obtained from Kparoun village, at Abomey-Calavi Commune to Atlantic Department were used.

The protocol [11 and 15] with modifications were used. Cubic pieces of 5 to 10 cm of tubers with the apex or axillary buds are cleaned with tap water added with liquid soap, then soaked in a fungal solution (Mancozeb) for 1 hour before being rinsed 4 times with distilled water. The explants (apical and axillary buds) were collected and immersed in 70% alcohol for 3 min, after immersed in a solution of mercury chloride (0.08%, 0.1% and 0,15%) with three immersion times 5min; 7min and 10min or sodium hypochlorite (8%, 10% and 12%) with three immersion times (25 min, 30 min and 45 min). The plant material is then rinsed three times with sterile distilled water within 5 minutes to remove all traces of disinfectant

Statistical analysis

The rates of infection, necrosis and survival were the parameters evaluated through daily observations on each explant cultivated. For each treatment, type of explant, dose of disinfectant and immersion time were evaluated with 5 repetitions. A total of 360 explants were therefore initiated for the 18 treatments.

In order to understand or predict the effect of different doses of NaOCl and HgCl2 on the behavior of apical and axillary buds at each immersion time, a binary logistic regression was performed. Also, analysis of variance was performed and averages were prioritized by the Student Newman and Keuls (SNK) test. Concerning the choice of the type of explant and the type of disinfectant, the test of comparison of two proportions performed.

RESULTS

Effect of sodium hypochlorite and mercuric chloride on the types of explants on infection

Table 1 shows the logistic regression of infection using sodium hypochlorite as a disinfectant. It revealed according to the probabilities associated with Khi2 tests ($p < 0.0001$) that explant interactions * NaOCl, explants * immersion time and NaOCl * immersion time were highly significant. This shows that NaOCl doses, immersion time and the type of explants cannot be dissociated to explain the infection of the explants. Thus, the doses of sodium hypochlorite and the immersion times applied. There was not infection affected the apical buds. On the other hand, the rate of infection of axillary buds varied from 0% (45 minutes) to 20% (25 minutes) at the dose of 8%. Using the dose of 10%, the infection rate also varied from 0% (45 minutes) to 10% (25 and 30 minutes). For the dose of 12% there was not infection observed for all immersion times applied (Fig 1). In addition, the discrimination coefficient is medium (R^2 = 46.54%) and the probability associated to the regression equation is very highly significant ($P < 0.0001$) (Table 1). The type of explants, NaOCl concentration and immersion time provide a quantity of information explained the appearance of infections, ie these variables can be used to discriminate the appearance of infections.

Table 2 shows the results of logistic regression of infection using mercuric chloride as a disinfectant. According to the probabilities associated with Khi2 tests ($p < 0.0001$), the type of explant interactions * dose of HgCl2, type of explants * immersion time and dose of HgCl2 * immersion time were highly significant. This showed that HgCl2 doses, immersion time and type of explant cannot be dissociated to explain the infection of the explants. Thus, at the dose of 0.08%, the infection rate varied from 20% (7 minutes) to 40% (5 minutes) with the apical buds. On the other hand, the infection rate of axillary buds, varied from 20% (7 minutes) to 80% (5 minutes). At the dose of 0.1%, only the immersion time of 5 minutes favored infection with an average rate of 20% with apical buds and 40% in axillary buds. At the dose of 0.15% there is not infection observed with the apical buds. The immersion time of 5 minutes favored an infection with an average rate of 20% in the axillary buds (Fig 2). The discrimination coefficient (R^2 = 46.54%) and the probability associated with the regression equation were very highly significant ($P < 0.0001$) (Table 4). The type explant, dose of HgCl2 and immersion time provides a quantity of information that can explain the appearance of infections, i.e. these variables can be used to discriminate the appearance of infections.

rate of infections.

Effect of sodium hypochlorite and mercuric chloride on the types of explants on necrosis

Table 3 shows the logistic regression results of necrosis using sodium hypochlorite. The probabilities associated with Chi^2 tests showed that the type of explant interactions * NaOCl dose, type of explant * immersion time were not significant respectively ($p = 0.7005$) and ($p =$

0.3069). There is an interaction between the NaOCl concentration and the immersion time (p = 0.0370). This showed that the doses of NaOCl and the immersion time cannot be dissociated to explain the necrosis of the explants in the tissue culture. Thus, for apical buds, no necrosis was observed for all immersion times applied at the dose of 8% of sodium hypochlorite. On the other hand, at the dose of 10%, the necrosis rate varied from 0% (25 minutes) to 10% (45 minutes). At 12% dose, the necrosis rate varied from 0% (25 minutes) to 20% (45 minutes). The necrosis rate varied from 0% (25 minutes) to 10% (45 minutes) of sodium hypochlorite) with the axillary buds. At 10% dose, the necrosis rate varied by 10% (25 minutes) at 40% (45 minutes) and at 12% dose, the necrosis rate varied from 20% (25 minutes) to 80% (45 minutes) (Fig. 3). Also, the coefficient of discrimination (R^2 = 14.39%) and the probability associated with the regression equation is significant (p <0.0001) (Table 3). The type of explant, NaOCl concentration do not provide sufficient information to explain the emergence necrosis, ie these variables cannot be used to discriminate the appearance of necrosis.

Table 1: **Effect of NaOCl and type of explants on infection: result of the binary logistic model**

Source	DDL	Khi² (Wald)	Pr > Wald	Khi² (LR)	Pr > LR	R² adjusted
Explant type	1	1,065E-06	0,9992	16100,5166	< 0,0001	
Dose of NaOCl	2	2,4279E-07	1,0000	28715,7953	< 0,0001	
Duration of immersion	2	2,1211E-07	1,0000	28715,7953	< 0,0001	
Explants*NaOCl	2	2,2963E-07	1,0000	28715,7953	< 0,0001	**0,465*** **
Explants* Duration of immersion.	2	2,5368E-07	1,0000	28715,7953	< 0,0001	
NaOCl * Duration of immersion	4	0,32185083	0,9884	28715,7953	< 0,0001	

Table 2: Effect of HgCl2 and the type of explants on infection: result of the binary logistic model

	DDL	Khi² (Wald)	Pr > Wald	Khi² (LR)	Pr > LR	R² adjusted
explants	1	10,1997171	0,0014	20,6876456	< 0,0001	
Dose of HgCl2	2	0,06724798	0,9669	2,37353957	0,3052	
Duration of immersion	2	1,39202197	0,4986	1,53329654	0,4646	
Explants * HgCl2	2	0,44970846	0,7986	4853,50506	< 0,0001	
Explorers * Duration of immersion	2	0,44976243	0,7986	22598,9189	< 0,0001	0,4654***
DoseHgCl2 * Duration of immersion	4	2,28599477	0,6833	22598,9189	< 0,0001	

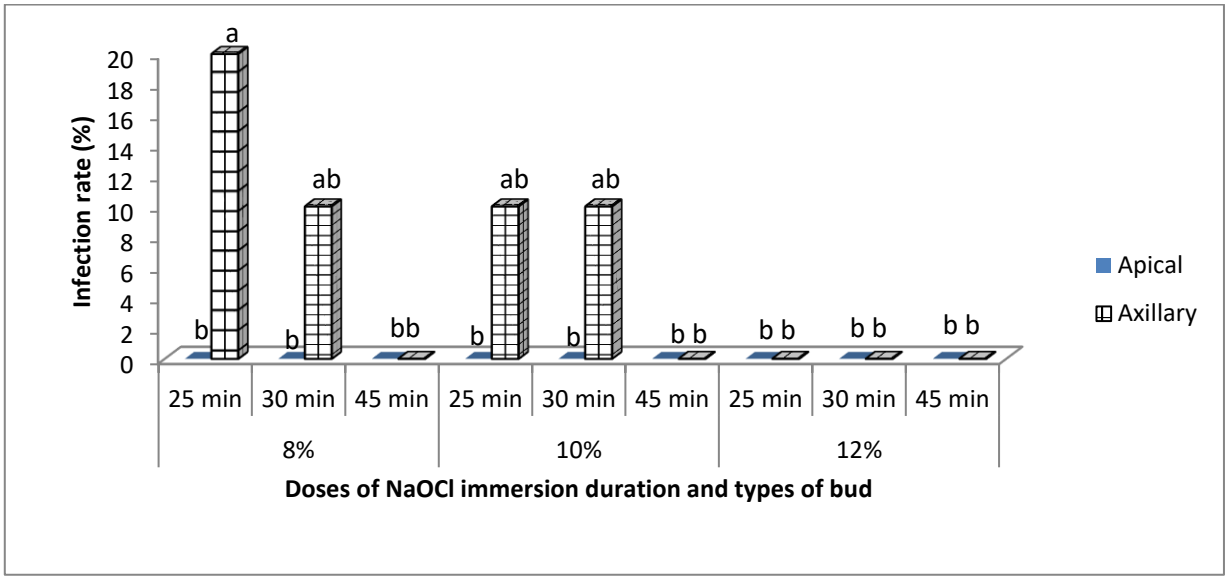

Figure 1: Mean infection rate by explant type of sodium hypochlorite dose and duration of immersion

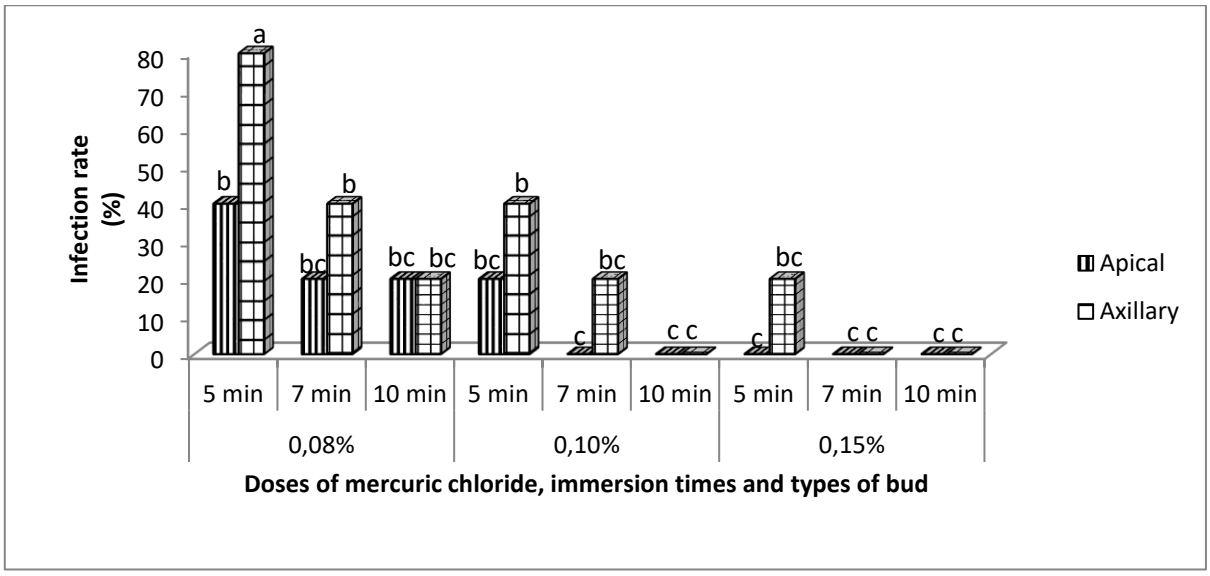

Figure 2: Average rate of bud infection based on mercuric chloride doses of immersion time and type of explant

Table 4 shows the logistic regression results of necrosis using mercuric chloride as a disinfectant. The probabilities associated with Khi^2 tests ($p < 0.0001$) showed that the type of explant interactions * dose of HgCl2, type of explants * immersion time and HgCl2 dose * immersion time are highly significant. This shows that HgCl2 doses, immersion time and type of explant cannot be dissociated to explain necrosis of explants in tissue culture. Thus, for the dose of 0.08% of mercuric chloride, the necrosis was not observed with the apical buds whatever the duration of immersion. On the other hand, using the axillary buds only the immersion time of 10 minutes presents necrosis with a rate of 40%. At the dose of 0.1%, only the immersion time of 7 minutes leads to necrosis of apical buds with an average rate of 20%. On the other hand, at the level of, the rate of necrosis varied from 40% (5 minutes) to 80% (10 minutes) with the axillary buds. At the dose of 0.15%, only the immersion time of 7 minutes leads to necrosis (20%) in the apical buds. The average rate of necrosis varied from 60% (5

minutes) to 100% (10 minutes) (Fig 4) with axillary buds. The discrimination coefficient (R^2 = 31.17%) and the probability associated with the regression equation is very highly significant (P <0.0001) (Table 4). The type of explant, HgCl2 concentration and immersion time don't provide a sufficient information to explain the appearance of necrosis.

Table 3: Effect of NaOCl and the type of explants on necrosis: result of the binary logistic model

Source	DDL	Khi² (Wald)	Pr > Wald	Khi² (LR)	Pr > LR	R² adjusted
Type of explants	1	0,364832	0,5458	0,3680995	0,5440	
Dose of NaOCl	2	3,20827001	0,2011	4,39829958	0,1109	
Duration of immersion	2	8,96791549	0,0113	12,150783	0,0023	
Explants * NaOCl	2	0,71100022	0,7008	0,71185292	0,7005	0,149***
Explorers * Duration of immersion	2	2,30270665	0,3162	2,36219603	0,3069	
NaOCl * Duration of immersion	4	8,26958141	0,0822	10,2118913	0,0370	

Table 4: Effect of HgCl2 and the type of explants on necrosis: result of the binary logistic mode

Source	DDL	Khi² (Wald)	Pr > Wald	Khi² (LR)	Pr > LR	R² adjusted
Explant type	1	7,4104E-05	0,9931	14,170727	0,0002	
Dose of HgCl2	2	0,19191486	0,9085	2121,3545	< 0,0001	
Duration of immersion	2	6,29545824	0,0429	5257,92384	< 0,0001	
Explants * HgCl2	2	3,1468825	0,2073	5257,92384	< 0,0001	0,311***
Explorers * Duration of immersion	2	0,03152419	0,9844	5257,92384	< 0,0001	
HgCl2 * Duration of immersion	4	7,57149053	0,1086	5257,92384	< 0,0001	

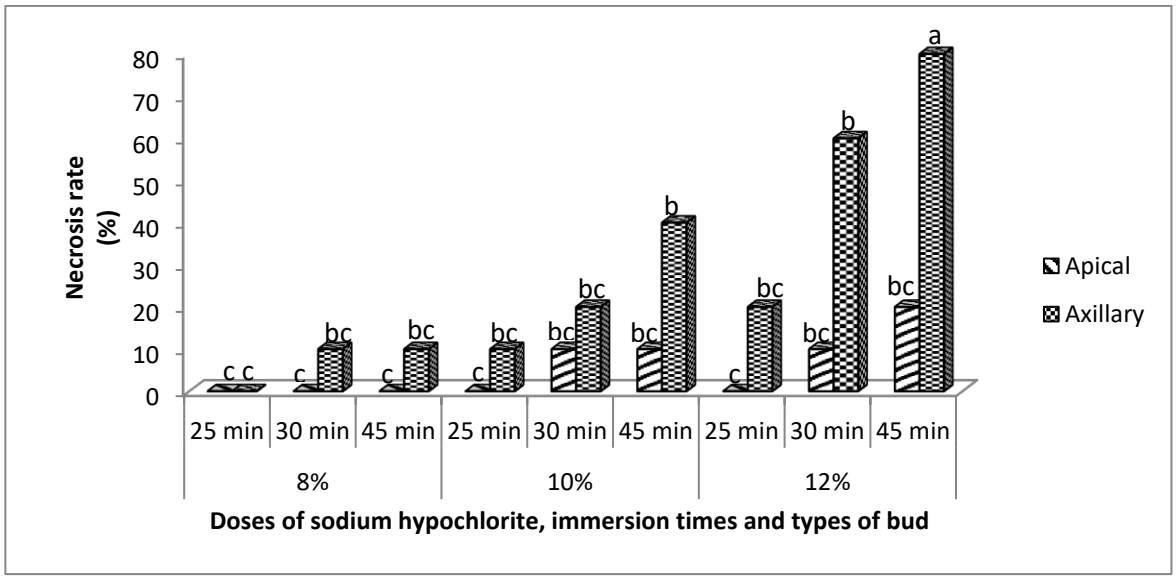

Figure 3: Average bud necrosis rate as a function of sodium hypochlorite dose of immersion duration and bud type

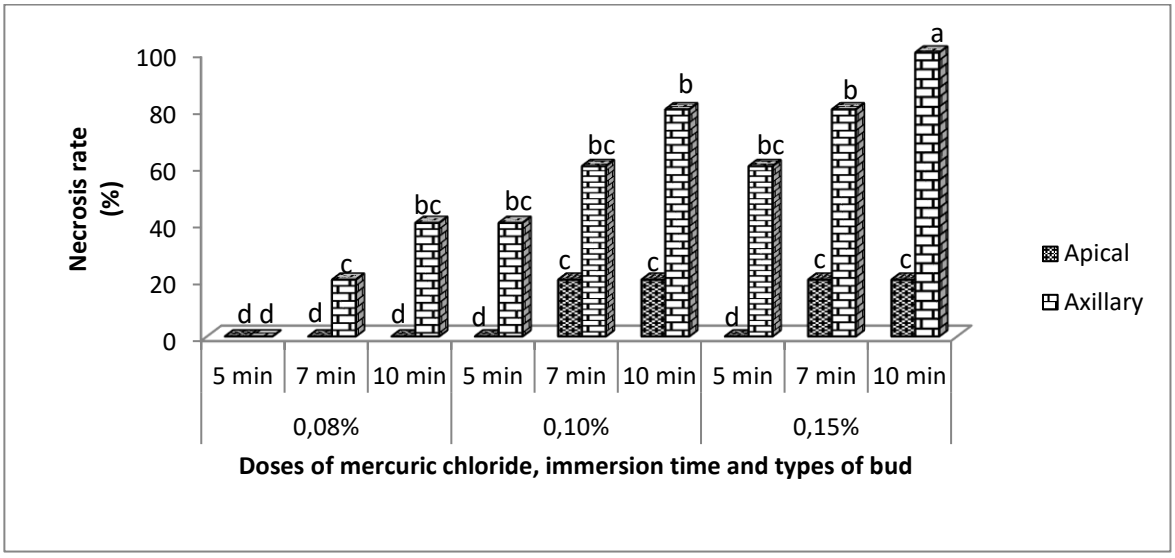

Figure 4: Mean explant necrosis rate based on mercuric chloride doses of immersion duration and bud type

Effect of sodium hypochlorite and mercuric chloride on the types of explants on survival

Table 5 showed the results of the logistic regression on survival using sodium hypochlorite as a disinfectant. According to the probabilities associated with Khi^2 tests ($p < 0.0001$) that the type of explant interactions * dose of NaOCl, type of explant * immersion time and dose of NaOCl * immersion time are highly significant. This showed that NaOCl doses, immersion time, and bud type cannot be dissociated to explain the survival the explants of taro. Thus, for apical buds, all axillary buds survived with a dose of sodium hypochlorite at 8% for all immersion times applied. On the other hand, for axillary buds at the dose of 8%, the survival rate varied from 80% (at 25 minutes and 30 minutes) to 90% (at 45 minutes). For 10% dose, the survival rate was from 90% (30 minutes and 45 minutes) to 100% (25 minutes). The survival rate varied from 60% (45 minutes) to 80% (25 minutes) in the axillary buds. For the 12% dose of sodium hypochlorite, the survival rate varied from 80% to 100% in the apical buds. The highest rate (100%) was observed with the immersion time of 25 minutes and the lowest rate (80%) is observed with the immersion time of 45 minutes. The survival rate varied from 20% (45 minutes) to 80% (25 minutes) with the axillary buds (Fig 5). The discrimination coefficient ($R^2 = 57.27\%$) and the probability associated to the regression equation is very highly significant ($P < 0.0001$) (Table 5). The type of explant, NaOCl concentration and immersion times provided much more information's to explain explant survival, ie these variables can be used to discriminate the survivorship of the explants.

Table 6 shows the results of logistic regression using mercuric chloride as a disinfectant. The probabilities associated with Khi^2 tests ($p < 0.0001$) that explant interactions * HgCl2 dose, type of explant * immersion time and dose of HgCl2 * immersion time were highly significant. This showed that HgCl2 doses, immersion times and explant type cannot be dissociated to explain survival of explants in tissue culture. Thus, for the dose of 0.08% of mercuric chloride, the survival rate varied from 60% (10 minutes) to 80% (7 minutes) with the apical buds. On the other hand, the rate of survival varied from 20% to 40% (7 minutes) with the axillary buds. At the dose of 0.1%, the survival rate is 80% for all immersion times applied to the apical buds and 20% to the axillary buds for these same immersion times. At the dose of 0.15%, the survival rate varied from 80% (7 minutes) to 100% (5 minutes) at

the level of the apical buds. A survival is achieved at the immersion time of 5 minutes with an average rate of 20% with the axillary buds (Fig 6). The discrimination coefficient (R^2 = 50.13%) and the probability associated with the regression equation is very highly significant (P <0.0001) (Table 6). The type of explant, HgCl2 concentration and immersion time provide a quantity of information (R^2 = 50.13%) to explain the survival of the explants, ie these variables can be used to discriminate the survival of the explants.

Table 5: Effet du NaOCl et du type d'explants sur la survie : résultat du modèle logistique binaire

Source	DDL	Khi² (Wald)	Pr > Wald	Khi² (LR)	Pr > LR	R² adjusted
Type of explants	1	7,4515E-05	0,9931	11,5663483	0,0007	
NaOCl dose	2	5,4072E-05	1,0000	2,41789846	0,2985	
Duration of immersion	2	0,49835363	0,7794	1198,08249	< 0,0001	
Type of explants * NaOCl	2	0,31221764	0,8555	2523,59765	< 0,0001	**0,572*** **
Type of explants * Duration of immersion	2	0,74049561	0,6906	2523,59765	< 0,0001	
NaOCl dose * Duration of immersion	4	1,36515995	0,8502	2523,59765	< 0,0001	

Table 6: Effect of HgCl2 and type of explants on survival: result of the binary logistic model

Source	DDL	Khi² (Wald)	Pr > Wald	Khi² (LR)	Pr > LR	R² adjusted
Dose of HgCl2	2	0,20122506	0,9043	638,106877	< 0,0001	
Duration of immersion	2	3,4841743	0,1752	2323,20672	< 0,0001	
Type of explants	1	5,15107244	0,0232	2323,20672	< 0,0001	
Dose of HgCl2 * Duration of immersion	4	2,1334068	0,7112	2323,20672	< 0,0001	0,5013****
Dose of HgCl2 * Explants	2	4,683E-05	1,0000	2323,20672	< 0,0001	
Immersion duration * Type of explants	2	1,5961E-05	1,0000	2323,20672	< 0,0001	

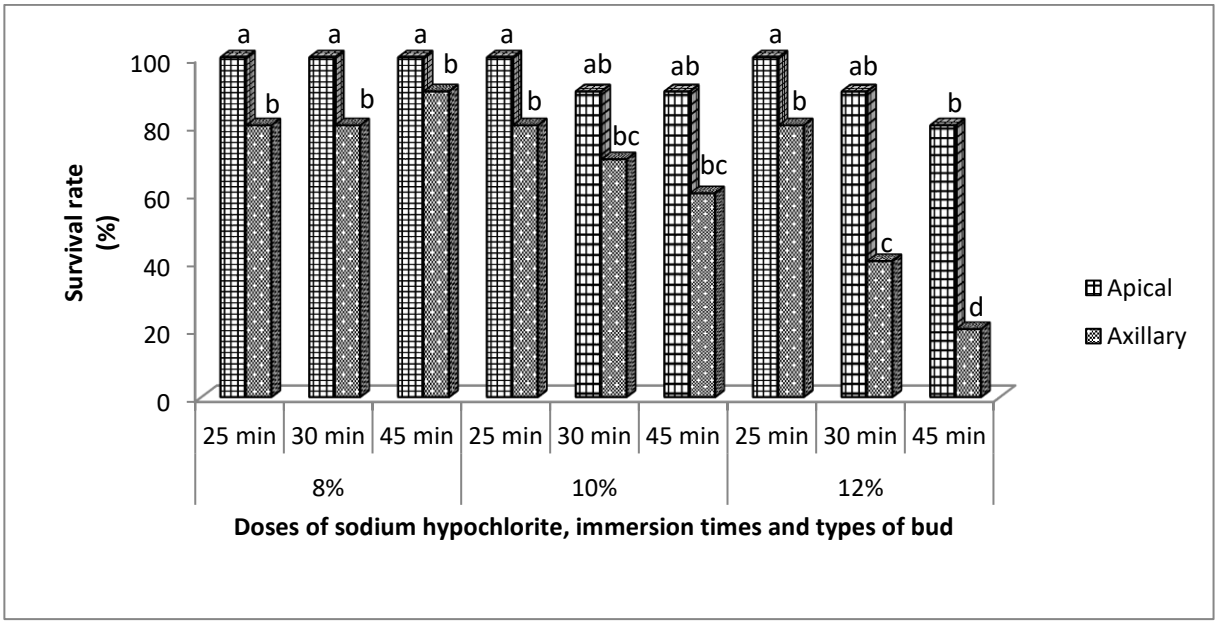

Figure 5: Mean survival rate of explants according to immersion times and type of explant

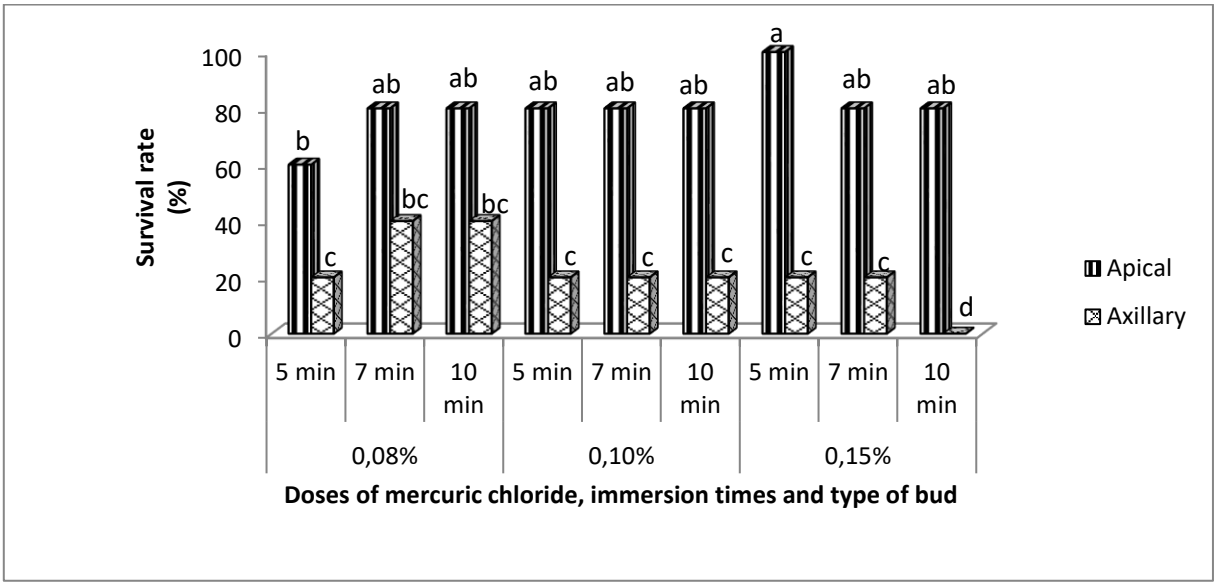

Figure 6: Survival rate of explants according to doses of mercuric chloride

Discussion

In view of these results, it is important to point out that the dose of disinfectant and the immersion time increase as well as the rate of infection decreases. Thus, the highest doses of sodium hypochlorite (12%) and mercuric chloride (0.15%) were requested to control infections. These results were similar with those obtained by [20] who showed that dose of 1% of mercuric chloride applied to yam explants can control infections. These observations were also consistent with the results of [21] who showed on sweet potato that a high dose of mercuric chloride helped to control infections in tissue culture. The difference of behavior in tissue culture observed between apical and axillary buds could be explained by the fact that the axillary buds are more infected because of their nudity, whereas the apical buds are protected by primordial leaves, which reduced the rate of infections.

The different doses of sodium hypochlorite (NaOCl) and mercuric chloride (HgCl2) showed a significant difference in infection, necrosis and survival of different types explants of taro. Thus, the axillary buds were more infected and necrotic than the apical buds for the different doses of NaOCl (8%, 10% and 12%) at all immersion times (25 min, 30 min and 45 min) and also for the different doses of HgCl2 (0.08%, 0.1% and 0.15%) at all immersion times (5 min, 7 min and 10 min). Similarly, the higher doses of both types of disinfectants caused a high rate of necrosis. These results were similar with those of [20] who showed on yams that more the dose of the disinfectant increases, more the necrosis rate increases. Similar results were also obtained by [21] whose attempts to disinfect explants of sweet potato with increasing doses of mercuric chloride observed necrosis of explants as the dose increased disinfectant increases. These same observations are made by [22] on teak microboutures (*Tectona grandis* L. f., Verbenaceae) with higher and higher doses of mercuric chloride (0.15% and 0.20%). The difference observed between the apical and axillary buds was explained by the nudity of the axillary buds. Indeed, the axillary buds being bare, they are penetrated by the disinfectant which results the cell death and necrosis of the buds. Apical buds are protected by primordial leaves, which reduced their penetration by the disinfectant [15].

The best survival rate (100%) for apical buds is obtained with 8% sodium hypochlorite dose for all immersion times (25 min, 30 min and 45 min), while the doses of 10% and 12% , the best survival rate was obtained for the

immersion time of 25 minutes. In the axillary buds, the best survival rate (90%) is obtained at the 8% dose of an immersion time of 45 minutes. For mercuric chloride, the best survival rate (100%) for apical buds was obtained with 0.15% dose at an immersion time of 5 minutes. For the axillary buds the best survival (40%) was obtained with the 0.08% dose for immersion times of 7 minutes and 10 minutes. The percentage of survival apical and axillary buds was the most important parameter to be considered when identifying the most appropriate dose of sodium hypochlorite, mercuric chloride, immersion time and type of explant for good disinfection [20]. These observations are contrary to those of [11] who obtained a survival rate of 43.8% for apical and axillary buds by disinfecting with 8% sodium hypochlorite with the respective immersion times of 45 min and 30 min. This could be explained by the no variation of the sodium hypochlorite dose and the immersion time. The percentage of survival was the most important parameter to consider when identifying the dose of sodium or mercuric chloride.

Conclusion

The study in vitro cultivation of taro (*Colocasia esculenta* L. SCHOTT) produced in Benin was part of a production dynamic and the conservation of local cultivars. The results generally showed that sodium hypochlorite at a dose of 8% with a 25-minute immersion time was favourable for disinfection of both apical and maxillary explants. On the other hand, for mercuric chloride, the results showed that the 0.1% and 0.08% doses were respectively effective for the in vitro survival of the apical and axillary explants for an immersion time of 5 minutes. As for the choice of material, apical explants were more resistant to infection and necrosis than axillary explants.

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